

HISTOPATHOLOGICAL TECHNIC

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Histopathological

KRAJIAN AND GRADWOHL

SECOND EDITION

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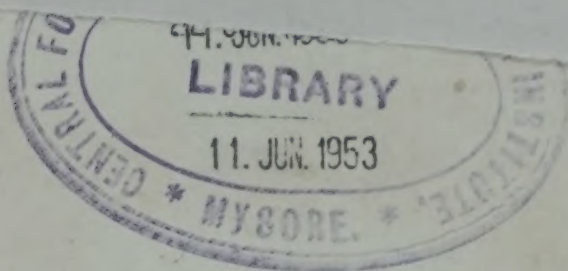
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HISTOPATHOLOGICAL TECHNIC



Histopathological Technic

Including a Discussion of Botanical Microtechnic

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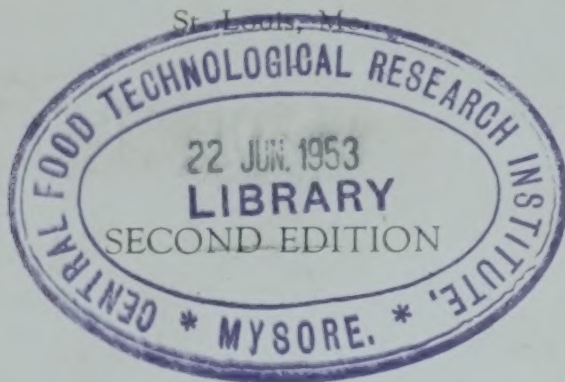
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*With 131 Text Illustrations
and 7 Color Plates*

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To the late

W. G. MACCALLUM, M.D.

Professor of Pathology and Bacteriology,
Johns Hopkins University Medical School

PREFACE TO SECOND EDITION

The second edition of Krajian's *Histological Technic* (1940) is intended, as was the first, for medical students, technicians, research workers, and laboratory directors. Specific directions are given concerning all the methods now used in microhistological applications. The latest improved methods are set forth so that the experienced worker may keep abreast of the times. In this edition the name of the book has been changed to *Histopathological Technic*.

The frozen section method and the preparation of permanent sections have been emphasized. The arguments in favor of the frozen section method are fully given, namely, rapid availability of examination and microscopical report.

The standard paraffin embedding methods utilizing the Autotechnicon are given with full details. This apparatus has now been accepted as standard equipment in most laboratories.

A new section on Use and Care of Microtome Knives by Lorimer Rutty, of Niagara Falls, N. Y., is included.

The methods incorporated in this book are those that the authors have found most rapid, economical, and practical. In the preparation of the book, valuable information has been obtained from the works or technics of Mallory and Wright, Carleton, McClung, and Hassin.

Special thanks are given to Dr. E. D. Woodhouse, who has prepared the section on Botanical Microtechnic; to Professor Cyril B. Courville, the well-known neuropathologist, who made many suggestions in arranging the methods and illustrations in the section on Neuropathological Methods.

This book contains, besides standard accepted methods, numerous original and modified methods such as methods of deformalinization, serumizing, complete description of frozen section method, dry ice method, complete details of Autotechnicon methods, Gradwohl's modification of Giemsa stain, xanthydrol reagent, Krajian's stains of aniline blue, reticulum, elastic fibers, amyloid, myelin sheath, new gram-positive and gram-negative stain on frozen and paraffin sections, rapid method for the demonstration of *Spirochaeta pallida* in frozen sections, rapid method for the demonstration of *Spirochaeta*

pallida in paraffin sections, new acid-fast stain, tumor reticulum for the differentiation of carcinoma from sarcoma, Fuller's acid-fast stain, Steiner's silver stain for microorganisms; cytological diagnosis of malignancy by Papanicolaou methods; and modification of Shu Chu Shen, botanical microtechnic, histopathological methods in the central nervous system, modified museum specimen preparation, and other numerous modifications of valuable methods.

It is the hope of the authors that the book will be found useful in the pathological laboratories of hospitals and research departments.

A. A. K.
R. B. H. G.

PREFACE TO FIRST EDITION

The degree of success in any manipulative branch of science is largely dependent on the worker's knowledge of hidden difficulties and their remedy.

In this handbook of histological technic many pitfalls have been freely indicated. This, I feel confident, will be helpful to the beginner and inexperienced worker who requires specific directions which he may apply to his problems to obtain the desired results; and to the experienced worker who seeks the latest improved methods.

The much neglected frozen section method in the preparation of permanent sections has been emphasized. Its chief advantages are rapidity, faithful preservation of normal structures of cell components, adaptability to many staining methods, and low cost. Because of these advantages it has been adopted as the routine section-cutting method in the histopathological laboratories of the Los Angeles County General Hospital.

The complete and accurate diagnosis of many lesions encountered in autopsy and surgical pathology often depends upon the availability of special staining methods. The use of such methods is often so hampered by the long delay involved in the preparation of microscopic sections (paraffin or celloidin), as generally described in microtechnics, that the diagnostic aids are omitted to the detriment of science and the patient.

A major purpose of this work is to present modifications of these valuable methods which make their results more quickly available

to the pathologist, often in a matter of minutes rather than hours or days, by the use of the frozen section procedure.

The book contains, besides the accepted standard methods, many new and improved methods; viz., the xanthydrol method for detecting excess urea in tissue sections and smears in uremia; a new method for staining *Spirochaeta pallida* in single tissue sections and smear preparations; a new aniline blue stain for connective tissue and hyaline on formaldehyde-fixed frozen and paraffin sections; a new method for demonstrating gram-negative organisms; a new mucin stain; a new method for serumizing loose texture tissues and body fluids; the eosinol method to replace the aqueous eosin as a counterstain in hematoxylin eosin stain; an improved scharlach R staining solution; an improved glycogen staining method; a method for impregnating fetal bony structure; an improved method for the preparation of museum specimens; the substitution of anhydrous isopropanol for absolute ethyl alcohol for dehydration purposes; a method to remove wrinkles from creased sections; a method to deformalinize tissues and sections; the use of identification numbers under cover slips for labeling sections; the inclusion of plant histological technic for the benefit of botanical students; and the inclusion of color plates.

This work is not intended to be a reference book. Only those methods have been incorporated which, through years of experience and experiments, we have found to be the most rapid, economical, and practical.

For convenience a detailed index has been prepared, listing methods under subject as well as under the author's name. Chemicals and reagents are also indexed.

In the preparation of this book I have gained valuable information from the works or technics of Mallory and Wright, Carleton, McClung, and Hassin.

It is a great pleasure and a distinct honor to acknowledge with sincere thanks my obligation to Professor W. G. MacCallum, of Johns Hopkins University, under whom I worked in the Department of Pathology of the Presbyterian Hospital of Columbia University, and whose cooperation has made this book possible. To thank him adequately would surpass my ability. Therefore, I dedicate this book to him. I am also greatly indebted to Professor Newton Evans, of the College of Medical Evangelists and Director of the Depart-

ment of Pathology of the Los Angeles County Hospital, under whose guidance and helpful suggestions I have gained much valuable information.

I am deeply indebted to Dr. E. D. Woodhouse, who has prepared the section on Botanical Microtechnic.

I wish to express gratitude to Professor Cyril B. Courville, the neuropathologist, for his kind suggestions in arranging the methods and illustrations in the section on neuropathological methods.

For the reading of the manuscript I am obligated to Doctors Albert F. Brown, William Knoll, and Aram Glorig.

I am indebted to Miss Alice Scott for her valuable technical assistance, and to Mrs. Ruth M. Bond for her painstaking secretarial work.

A. A. KRAJIAN

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Histopathological Technic

PART I

FIXATION OF TISSUE

To preserve the normal structure of the histological material, it is essential to fix and harden the tissue as soon as it is removed from the body, either during life or after death.

The immediate fixation of the tissue not only preserves the cells and intracellular substances, but also prevents the post-mortem changes that take place after death due to putrefactive action of bacteria.

The chief aim of the fixation is to cause coagulation or precipitation of protoplasmic substances, which renders the cells and tissue elements resistant to such further chemical effects to which they are subjected before microscopic sections are prepared. Some fixatives, such as Zenker's fluid, act as mordants, which cause a particular staining reaction. Other fixatives, such as alcohol or isopropanol, have a hardening effect.

A well-fixed tissue forms the foundation for satisfactory section-cutting and staining. Tissues removed for microscopic study should be cut in small, thin blocks, and placed in a chosen fixing solution about 25 times their volume. If formaldehyde is used, a 15 per cent solution is employed during the first 24 hours. They are then properly trimmed into square or rectangular blocks and placed in 10 per cent solution, which is changed after 24 hours. Zenker's and other chrome salt solutions need not be changed.

To fix the eye specimens after enucleation, inject the tissue with a 10 per cent solution of formaldehyde and place it in a similar solution for 10 days before trimming for microscopic section.

Tissues previously fixed in alcohol can be washed under running water for 15 minutes, then placed in 10 per cent formaldehyde for 24 hours, and frozen sections cut.

Fixing Reagents and Solutions

A good fixative is one that penetrates, kills, fixes, and hardens the tissues so quickly that their structure is not altered in the process of dehydration, embedding, staining, clearing, and mounting.

The choice of a proper fixing reagent depends on the nature of the pathological lesion present in the tissue. Usually several reagents are combined in a given fixative in order to increase its power of penetration, so that both the cell and tissue components are properly fixed.*

All types of fixatives are not compatible with all the staining methods; some fixing reagents act as mordants for a particular staining process, while others inhibit certain stains.

The following fixatives allow most of the staining:

Bouin's solution.

Zenker's solution.

Bichloride-formaldehyde combination.

Mercuric chloride.

Carnoy fluid.

Formaldehyde solution prevents carmine stains.

Alcohol is followed by weak-staining stains other than carmine stains.

Regaud's solution stains poorly with carmine.

Osmic acid prevents carmine stains.

A stock solution of the following fixing solutions should be kept on hand. The amount of each depends on the quantity used in the laboratory. Generally, most of the fixing solutions are stable.

Zenker's Fluid

Among the many fixing reagents, Zenker's fluid, after many years of constant trial, stands out as probably the most reliable and effective. Zenker-fixed tissues preserve nuclear structure, bacteria, and fibrils of all kinds.

Mercuric chloride -----	5	Gm.
Potassium bichromate -----	2.5	Gm.
Sodium sulfate -----	1	Gm.
Distilled water -----	100	c.c.
Glacial acetic acid -----	5	c.c.

Zenker's fluid does not keep well in acetic acid. Therefore, prepare a stock solution of Zenker's fluid and add the acetic acid immediately before using. Fix tissues 12 to 16 hours. Wash overnight under a gentle stream of tap water to remove all the excess

*Note: Application of a 5 per cent potassium ferrocyanide solution to the section of degenerated tissue will help to regain the greater part of the staining of the nuclei of the cell.

potassium bichromate, and transfer to 70 per cent alcohol. Allow them to remain in 70 per cent alcohol until they are ready to be dehydrated, cleared, and embedded by one of the embedding processes.

Helly's Fluid

Helly's fluid is a slight modification of Zenker's fluid. Helly omitted the use of glacial acetic acid and replaced it with a 10 per cent solution of formaldehyde, which is added to the mixture at the time of use.

Mercuric chloride -----	5 Gm.
Potassium bichromate -----	2.5 Gm.
Sodium sulfate -----	1 Gm.
Distilled water -----	100 c.c.
Formaldehyde (40 per cent) -----	10 c.c.

Fix tissues for 8 to 16 hours. Wash for 12 to 16 hours under gentle stream of water, and transfer to 70 per cent alcohol. Allow to remain until ready to embed.

Regaud's Formol Bichromate Fluid

Potassium bichromate (3 per cent) -----	80 c.c.
Commercial formaldehyde (40 per cent) -----	20 c.c.

Regaud's solution gives excellent results with mammalian tissues. It costs less and penetrates better than chromosmium fixatives.

The solution is unstable and should be prepared fresh at the time of use. Fix tissues for 4 days, changing the fluid every day. Wash in running water for 16 to 24 hours, and transfer to 70 per cent alcohol.

Formaldehyde ✓

Formaldehyde is a gas, soluble in water to the extent of 40 per cent by weight.

Formaldehyde has been in general use for many years as a fixing reagent. It penetrates and fixes the tissues quickly, thereby enabling one to cut and stain satisfactory frozen sections. Formaldehyde preserves fat, myelin, nerve fibers, amyloid, hemosiderin, and various organisms. The best strength is a 10 per cent solution, which is prepared by taking one volume of commercial (40 per cent) formaldehyde to nine volumes of tap water or saline. To prevent formaldehyde from developing formic acid, which is injurious to

the tissue, the stock solution of formaldehyde should be neutralized by addition of 10 Gm. of calcium carbonate, lead oxide, or bicarbonate of soda, to a gallon of fluid. When specimens are to be kept for long periods, it is essential to place a few marble chips in the container.

Formaldehyde vapor is very injurious to the eyes, nasal mucosa, hands, and other exposed parts of the body, and contact should be avoided as much as possible. Allergic people may develop formaldehyde dermatitis, which may become very serious. The lesions persist for months.

The following solution will relieve and heal the lesion caused by formaldehyde, in course of time:

Urea crystals C.P. -----	5 Gm.
Ammonium phosphate C.P. -----	1 Gm.
Distilled water -----	100 c.c.

For protozoological work, fixation in Bouin's or Schaudinn's solution is preferable.

Picric Acid

Picric acid is a coagulant of protoplasm and is used as a saturated aqueous solution. Its solubility is between 0.5 and 1 per cent at room temperature.

Bouin's Fluid

Picric acid (saturated aqueous solution) -----	75 c.c.
Formaldehyde (40 per cent commercial) -----	25 c.c.
Glacial acetic acid -----	5 c.c.

This solution keeps well and has considerable penetrating power. It causes very little shrinkage. Picric acid, which stains the tissue yellow, is extracted in the process of dehydration.

It is not a good fixative for cytological work or inclusions such as Golgi bodies and mitochondria, because they are distorted or dissolved. It is a good fixative for small skin lesions.

Schaudinn's Solution

Mercuric chloride (saturated aqueous solution) --	2 parts
Absolute, or 95 per cent, alcohol -----	1 part
Glacial acetic acid in proportion of 10 per cent	

Alcohol

Alcohol is a fixative that both hardens and dehydrates tissue at the same time. It also preserves bacteria, fibrin, pigments, and elastic fibers, and is the only reagent (absolute alcohol) to preserve glycogen.

Tissues to be kept for a considerable period in alcohol should be placed in dilute (70 per cent) alcohol. If left longer than 48 hours in absolute alcohol, they become so hard that section-cutting is almost impossible.

The material fixed in alcohol should not be over 0.5 cm. thick. Strong alcohols, 95 per cent or absolute, harden only the surface of the tissue, so that deeper parts are not penetrated. To avoid this, a 70 per cent alcohol, gradually increased to 95 per cent, should be used.

Isopropanol

In recent years anhydrous isopropanol has been successfully substituted for absolute ethyl alcohol as a dehydrator in the preparation of paraffin blocks and staining procedure. It is recommended for its great economy and may be obtained through any laboratory supply house.* It must be understood, however, that it cannot be used successfully in the preparation of alcoholic solutions, such as acid alcohol, ether-alcohol mixture for dissolving celloidin, or alcoholic dye solutions.

Bichloride of Mercury and Formaldehyde Combination

A saturated solution of bichloride of mercury, when used alone, causes serious shrinkage of the cells. A combination with formaldehyde has been found to be a very useful fixing reagent.

Bichloride of mercury (saturated aqueous solution—8 Gm. to 100 c.c. water)	----- 90 c.c.
Formaldehyde (40 per cent commercial)	----- 10 c.c.

Fix thin pieces of tissue from 6 to 12 hours, place in 70 per cent alcohol, dehydrate, and embed in the usual manner.

Osmic Acid Solutions

Osmic acid is a very expensive reagent and is sold in sealed glass tubes of 0.5 and 1.0 Gm. The solution is prepared by dissolving 1

*Union Carbide and Carbon Co. product has been found superior.

Gm. of osmic acid crystals in 100 c.c. of pure, dust-free distilled water. The solution of osmic acid is easily spoiled by formation of black precipitates. This can partially be remedied by the addition of a few drops of a 10 per cent chromic acid or a 10 vol. of hydrogen peroxide. The vapor of osmic acid is irritating to the eyes.

Osmic acid is used to demonstrate fat, but its penetrating power is poor. For this reason, Flemming and Marchi have combined osmic acid with other reagents, such as chromic acid and bichromate of potash, to increase its penetrating power.

Flemming's Solution

Osmic acid (2 per cent aqueous solution) -----	20 c.c.
Chromic acid (1 per cent aqueous solution) -----	75 c.c.
Glacial acetic acid -----	5 c.c.

Fix thin blocks of tissue, not over 2 mm. thick, for 12 to 24 hours. Wash under a gentle stream of water overnight, place in 70 per cent alcohol, dehydrate, and embed in the usual manner.

The stock solution keeps poorly; therefore, it is best to keep osmic and chromic acid solutions in separate bottles and mix them at the time of use. The amount of the solution should be about 5 times the volume of tissue.

Marchi's Fluid

Bichromate of potassium -----	2.5 Gm.
Sodium sulfate -----	1 Gm.
Water -----	100 c.c.

Mix two parts of the above solution with one part of osmic acid (1 per cent aqueous solution).

Fix thin blocks of tissue, not over 2 mm. thick, in the above solution from 5 to 8 days. Wash under running water overnight, place in 70 per cent alcohol, dehydrate, and embed in the usual manner.

The stock solution is not stable; therefore, these solutions should be mixed at the time of use.

Potassium Dichromate

Potassium dichromate forms insoluble compounds with cell proteins and lipoidal bodies. A 3 per cent solution in distilled water is employed. Its saturation point is about 12 Gm. in 100 c.c. distilled water.

Xanthydrol Reagent

Acetic acid combined with xanthydrol is used to preserve and demonstrate urea crystals in tissue and smears.

Xanthydrol crystals	-----	5 Gm.
Glacial acetic acid	-----	100 c.c.

Fix blocks of tissue, not over 2 mm. thick, for 6 hours cold, or 1½ hours at 70° C. Wash under running water for 5 minutes, and place in 10 per cent cold formaldehyde for 12 hours, or ½ hour at 70° C.

Deformalinization

Technic to Remove Formalin From Formol-Fixed Sections and Tissues

At times it becomes necessary to refix formol-fixed tissues or sections in some other fixative, such as Zenker's fluid, for special stains. The following technic has given us satisfactory results in the majority of cases.

For Sections

1. Deformalinize for 1 hour in ammonia water (40 drops of strong ammonium hydroxide to 100 c.c. of water).
2. Wash for 1 hour in running water.
3. Fix for 1 hour in Zenker's or Helly's fluid.
4. Wash for 1 hour in running water.
5. Stain with desired method.

For Blocks

1. Deformalinize for 2 days in oven at 40° C. in ammonia water (40 drops to 100 c.c. of water).
2. Wash for 24 hours in running water.
3. Fix in Zenker's fluid for 12 hours or in Helly's fluid for 5 hours.
4. Wash for 12 hours in running water.
5. Place in 70 per cent alcohol until ready to embed.

Acetic Acid

In pure form it is called glacial acetic acid and is mixed with numerous fixatives and stains in various amounts.

Acetone

Acetone as a fixative has limited application. It is used as a rapid fixing solution of brain tissue for the diagnosis of rabies and as a fixative for enzymes.

Susa Fixative

Mercuric chloride -----	4.5 Gm.
Sodium chloride -----	0.5 Gm.
Trichloracetic acid -----	2.0 Gm.
Glacial acetic acid -----	4.0 c.c.
Formaldehyde -----	20.0 c.c.
Distilled water -----	80.0 c.c.

Place the tissue in Susa fixative for 5 to 48 hours, depending on the size.

Wash in tap water 3 to 5 hours, changing the water frequently. Place in 50 per cent alcohol for half a day, changing the alcohol several times.

Transfer to 70 per cent alcohol for half a day, changing several times.

Transfer to 95 per cent alcohol for half a day, changing alcohol several times.

Place in absolute alcohol not longer than 1 hour.

Clarify in pure xylol. The tissue must become transparent.

Embed in usual manner.

This fixative is used for sections to be stained by Giemsa stain (Schilling method, modified by Gradwohl).

DECALCIFICATION

Bone tissues to be decalcified should be sawed with a fine saw into thin slices so that they will decalcify quickly. Very dense and hard bones should not be more than 2 or 3 mm. thick; softer tissues do not need to be thinner than 4 to 6 mm.

Bones and calcareous tissues are thoroughly fixed in alcohol-formalin mixture (10 parts of formaldehyde in 90 parts of 95 per cent alcohol) or in Zenker's fluid. They should be left in alcohol-formalin solution for 2 to 4 days or in Zenker's fluid from 16 to 24 hours before they are submitted to the decalcifying process.

After decalcification, remove acid with a weak solution of lithium carbonate or sodium sulfate, or any suitable neutralizing reagent,

and place in 70 per cent alcohol or isopropanol to prevent further swelling. For the preparation of frozen sections, remove from 70 per cent alcohol, and fix in 10 per cent formaldehyde for 24 hours.

Formic, nitric, hydrochloric, acetic, picric, sulfurous, trichloroacetic, and sulfosalicylic acids and others are used alone, or in combination with other chemicals, for decalcification purposes.

Of all the reagents, formic, trichloroacetic, and nitric acids are the most useful and important. Formic acid combined with trichloroacetic acid acts quickly without swelling of the tissues, and does not interfere with any of the staining processes. The nuclei of the cells stain beautifully with the hematoxylin-eosin method. Nitric acid also acts quickly and without much swelling, but affects the staining property of the cell, and often the nuclei fail to retain the blue hematoxylin stain.

Formic Acid Method

(Evans and Krajian Method)

Bone tissues, properly fixed in alcohol-formalin fixative, are placed in the following decalcifying fluid:

Formic acid, C. P., 85 per cent	1 part
Alcohol, 95 per cent, or isopropanol	1 part
20 per cent sodium citrate in 1 per cent trichloroacetic acid	1 part

This mixture keeps well.

Nitric Acid Method

Decalcify fixed bone tissues in the following fluid, changing the solution every 24 hours until decalcification is complete.

Formaldehyde	10 c.c.
Nitric acid	5 c.c.
Distilled water	85 c.c.

When the tissues are completely softened, place them in a 5 per cent sodium sulfate or 2 per cent lithium carbonate solution 6 hours or longer to remove acid. They are then ready to be sectioned by one of the standard methods.

PART II

EQUIPMENT FOR SECTION-CUTTING

TYPES OF MICROTOMES

There are many types and makes of microtomes on the market. After many years of constant use and trial, we have found Bausch & Lomb microtomes mechanically simple but accurate, and very easy to operate, with satisfactory results.*

The rotary type microtome (Figs. 2 and 3) is used exclusively for cutting paraffin sections.

The sliding microtome (Figs. 4 and 5) is used for special purposes, principally for cutting celloidin and large, refractory paraffin material. In this microtome the knife moves against the object, a slow but very steady motion being required to manipulate the instrument.

The freezing microtome (Fig. 1) is used exclusively to cut unembedded, formalin-fixed tissues, which are frozen to the proper consistency with carbon dioxide.

Freezing Microtome

(Fig. 1)

The freezing microtome, manufactured by Bausch & Lomb Optical Co., is clamped to the bench.

1. The knife is mounted on a sliding arm and tightened with two screws at an angle of about 45 degrees.
2. Directly under the knife is the block holder.
3. Screw to tighten the block holder.
4. Ratchet-feed wheel.
5. Small valve to let carbon dioxide gas into chamber of block holder.
6. Copper feed pipe connecting carbon-dioxide gas to microtome.
7. Flywheel.
8. Staining dishes.
9. Large Pyrex test tube for boiling formalin.

*The Spencer instruments have been equally successful in our hands (R. B. H. G.).



Fig. 1.—Freezing microtome.

10. Cylinder containing 50 pounds of liquid carbon dioxide.
A tank, using solid carbon dioxide, or dry ice, is now available.
11. Wooden stand for cylinder.

This microtome is operated by the rotation of the flywheel, causing a reciprocating motion of the knife over the block, the thickness of the section being automatically regulated by ratchet-feed wheel.

Paraffin Microtome

(Small Type)

(Fig. 2)

(Manufactured by Bausch & Lomb Optical Co.)

1. Metal block holder.
2. Block holder locking screw.
3. Micrometer adjustment for thickness of section.
4. Ratchet-feed wheel.
5. Knife-holder screws.
6. Clamp nuts for knife stand regulator.
7. Knife stand clamp nut.
8. Pillar locking pin.
9. Flywheel.

This microtome is operated in the same manner as the freezing microtome.

Paraffin Microtome

(Large Type)

(Fig. 3)

(Manufactured by Bausch & Lomb Optical Co.)

1. Metal block holder.
2. Block holder locking screw.
3. Micrometer adjustment for thickness of section.
4. Pawl or catcher of ratchet-feed wheel.
5. Ratchet-feed wheel.
6. Knife-holder screws.
7. Clamp nuts for knife stand regulator.
8. Knife stand clamp nut.
9. Metal cover for microtome.
10. Pillar locking pin.
11. Flywheel.

This microtome is operated in the same manner as the small type paraffin microtome.

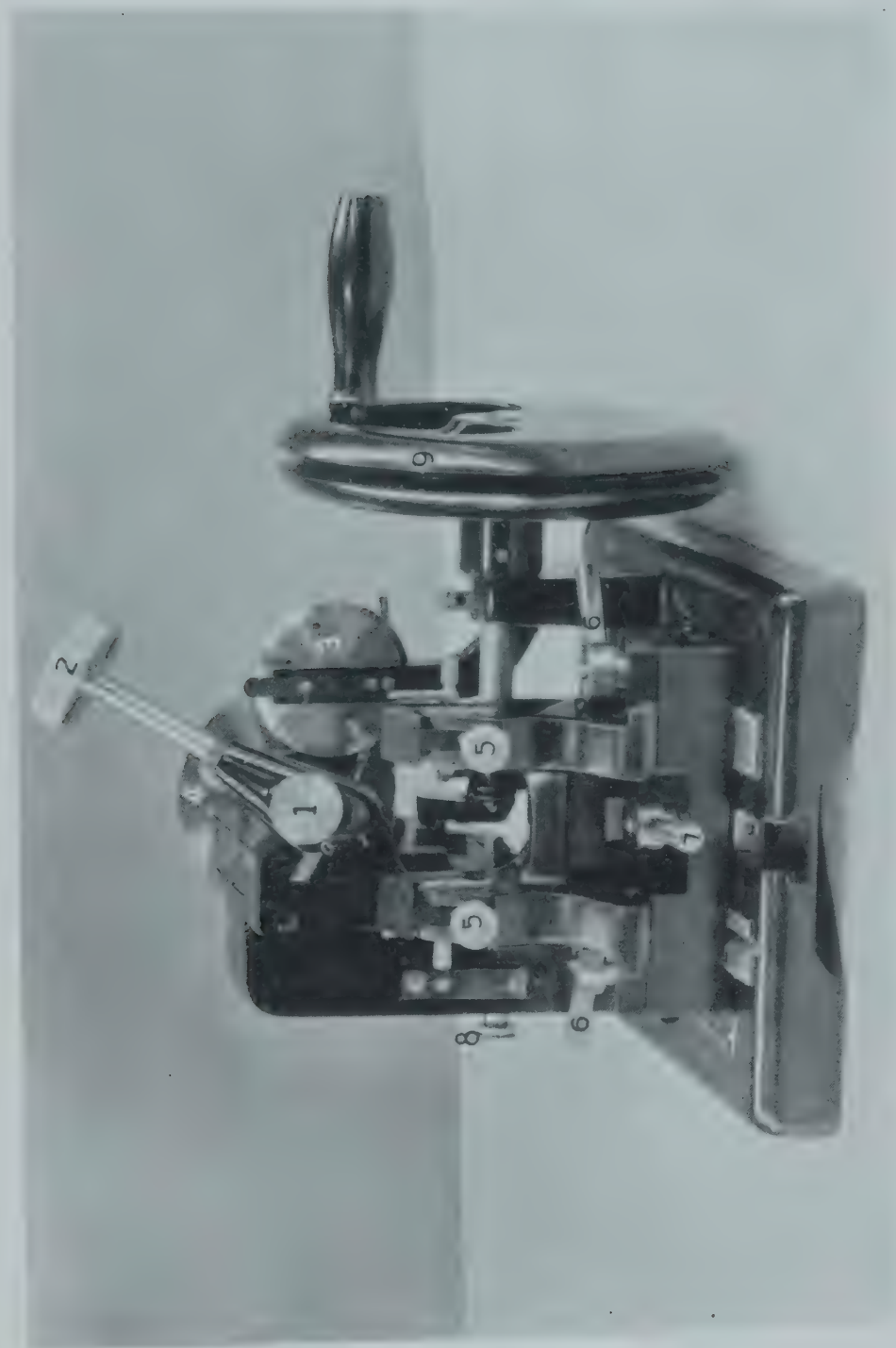


Fig. 2.—Paraffin microtome (small type).

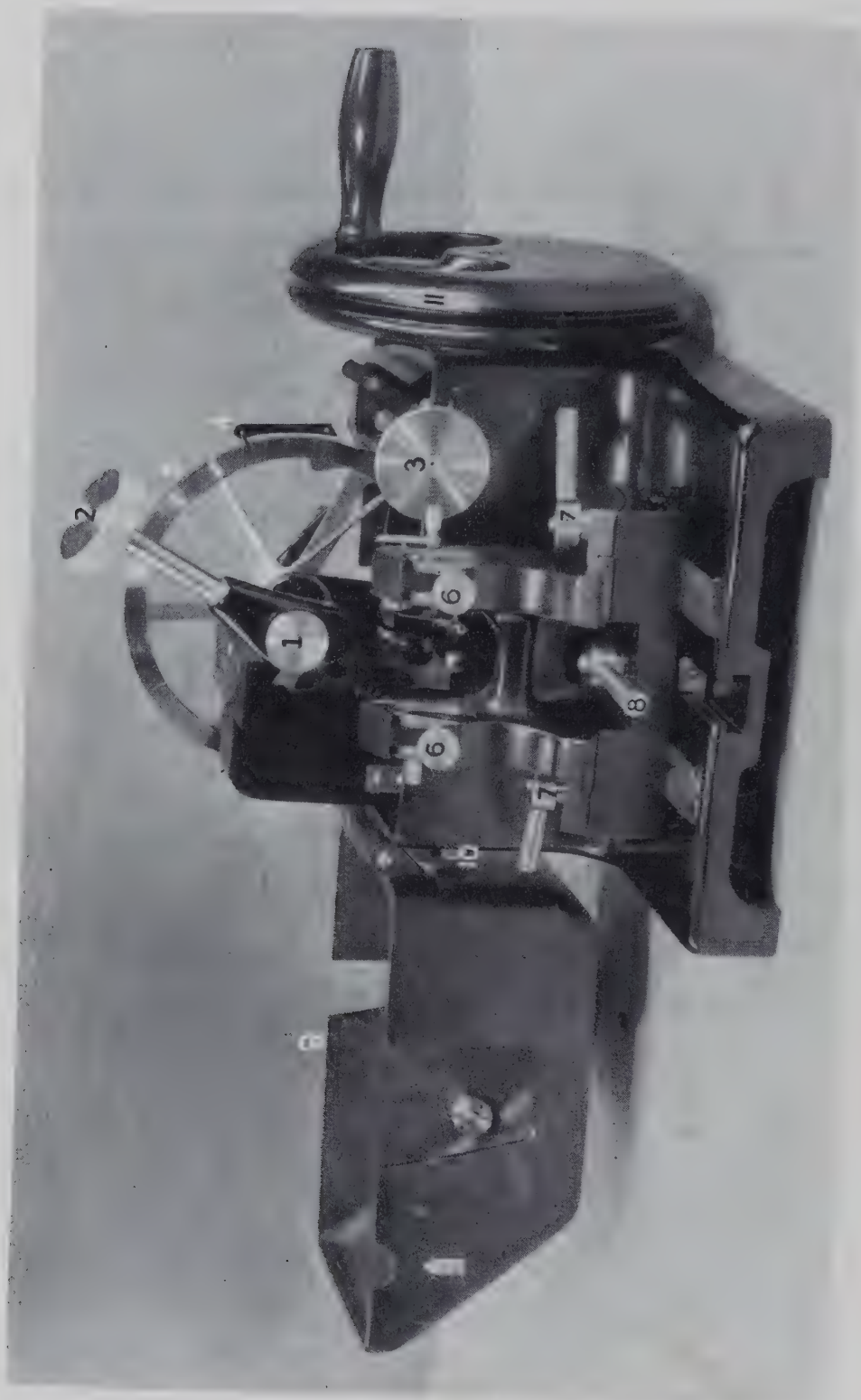


Fig. 3.—Paraffin microtome (large type).

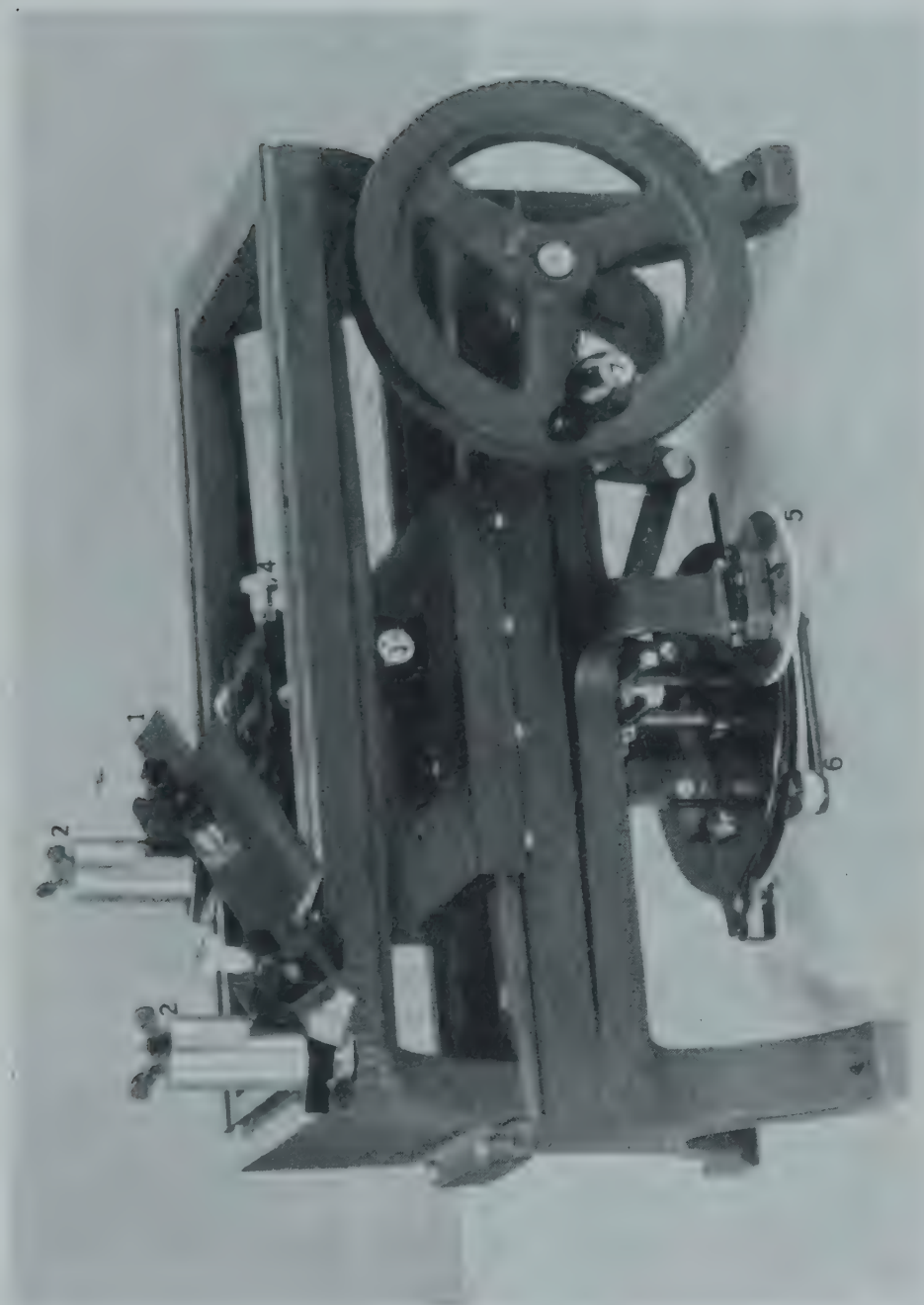


Fig. 4.—Colloidin microtome.

Celloidin Microtome

(Figs. 4 and 5)

(Manufactured by Bausch & Lomb Optical Co.)

1. The knife.
2. Knife-holder screws to regulate the angle of the knife.
3. Screw to regulate the block holder.
4. Screw to regulate the angle of the block holder.
5. Micrometer adjustment for thickness of the section.
6. Ratchet-feed wheel.
7. Flywheel.

This microtome is operated in the same manner as the paraffin microtomes.

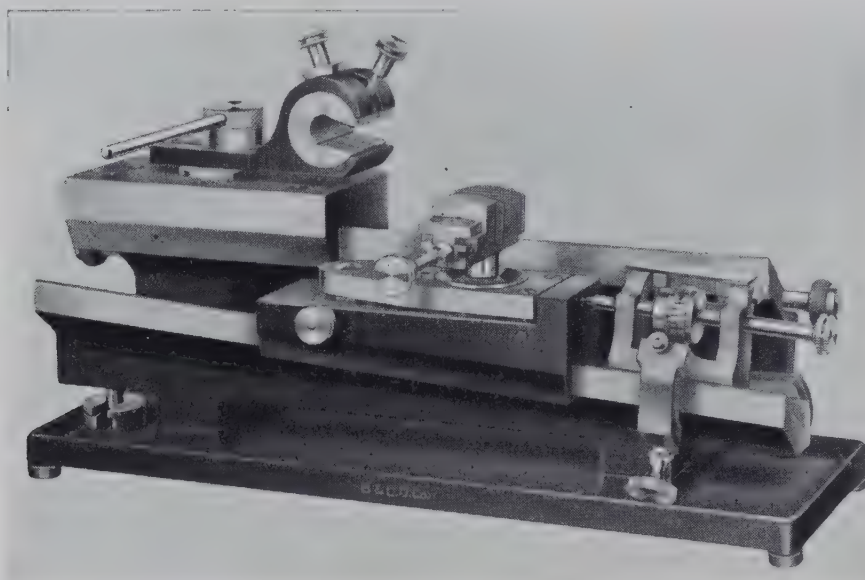


Fig. 5.—New type celloidin microtome. (Manufactured by Bausch & Lomb Optical Co.)

Micron

The micron is the standard unit of measurement in microscopy. One micron is $\frac{1}{1,000}$ of a millimeter, or $\frac{1}{25,000}$ of an inch.

MICROTOME KNIVES

There are three types of microtome knives:

1. The plane-wedge knife used for cutting frozen sections.
2. The biconcave type, used for paraffin work with the rotary microtome.

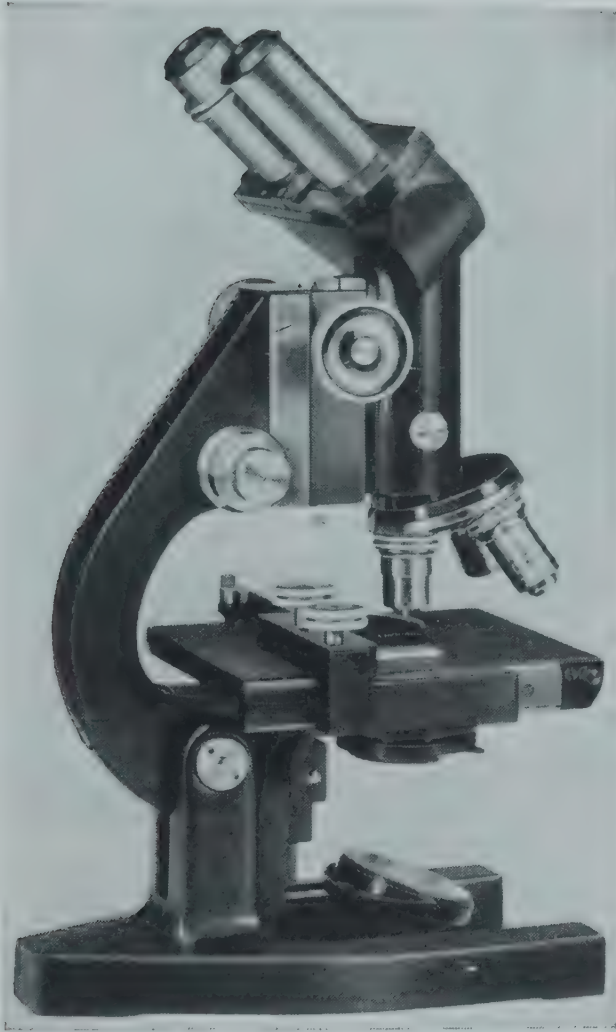


Fig. 6.—Binocular microscope, with 2 eyepieces, coarse and fine adjustments, 3 lenses, built-in mechanical stage, and mirror. (Courtesy Bausch & Lomb Optical Co.)



Fig. 7.—Microscopic substage lamp. (Courtesy Bausch & Lomb Optical Co.)



Fig. 8.—Microscope lamp, adjustable, with spherical condenser and 100 watt Mazda bulb. (Courtesy Bausch & Lomb Optical Co.)

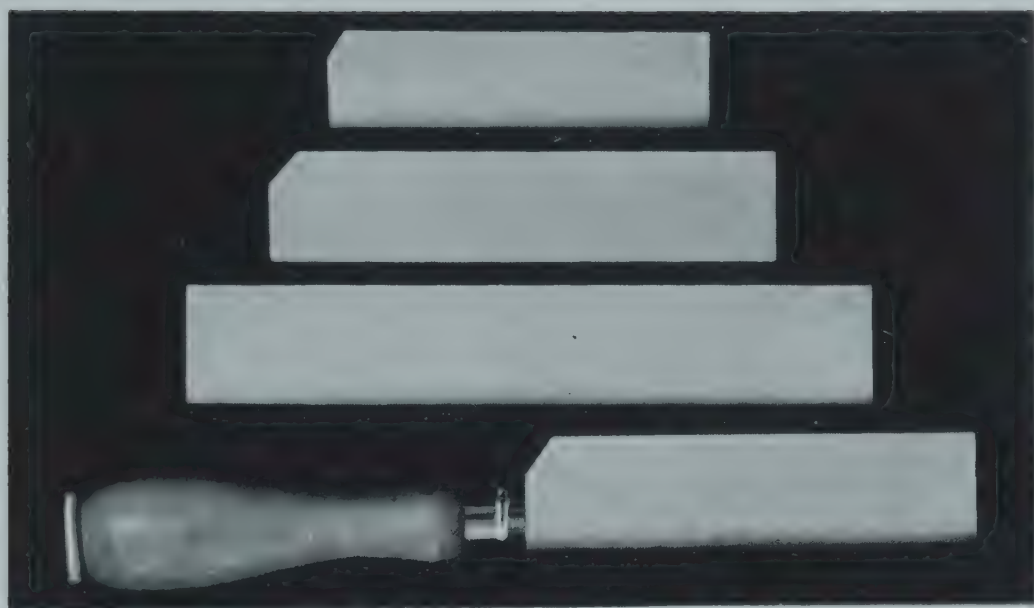


Fig. 9.—Microtome knives, various sizes, one with handle attached. (Courtesy Bausch & Lomb Optical Co.)

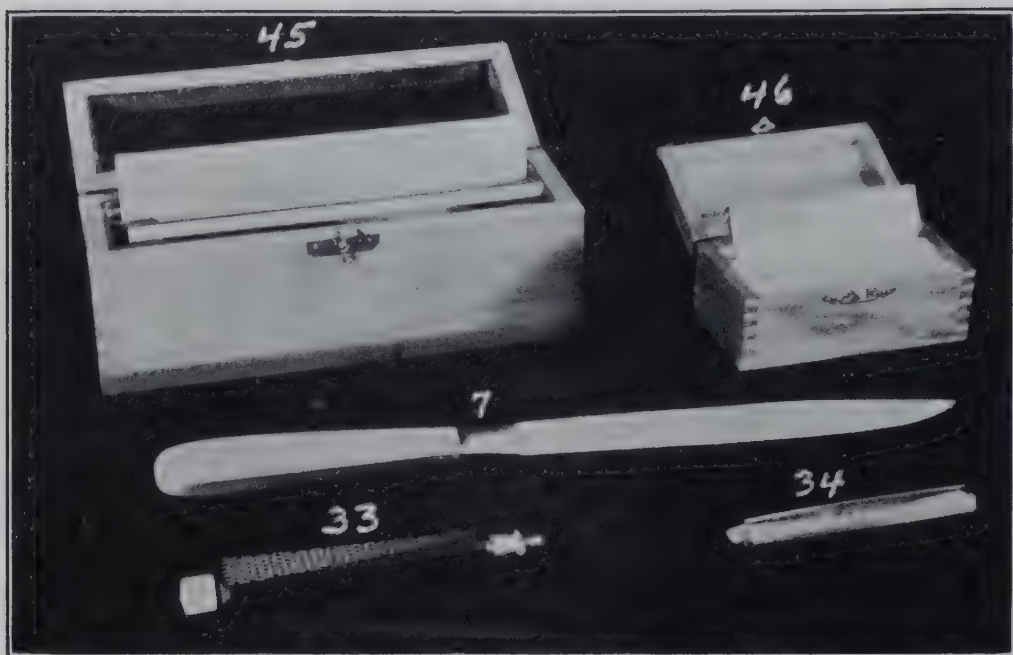


Fig. 10.—7, Large knife for trimming tissue blocks. 33, Wooden handle for microtome knife. 34, Metal back for microtome knife. 45, Paraffin microtome knife and box. 46, Freezing microtome knife and box. (Microtome knives, handle, and back, manufactured by Bausch & Lomb Optical Co.)

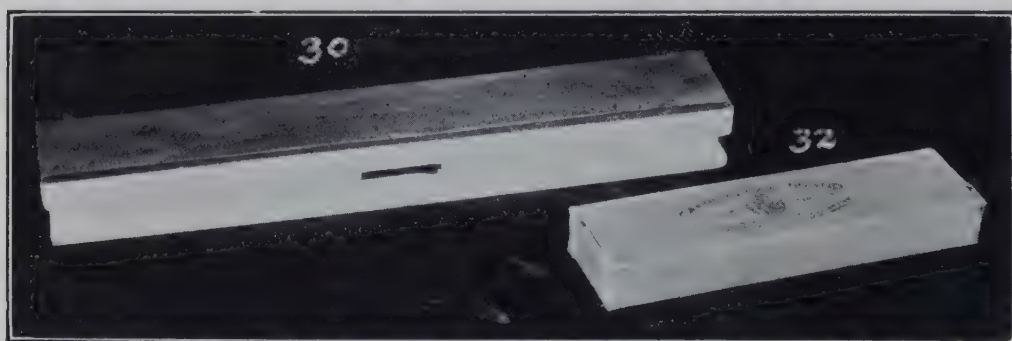


Fig. 11.—30, Leather strop to sharpen knives. 32, Carborundum stone to sharpen knives.

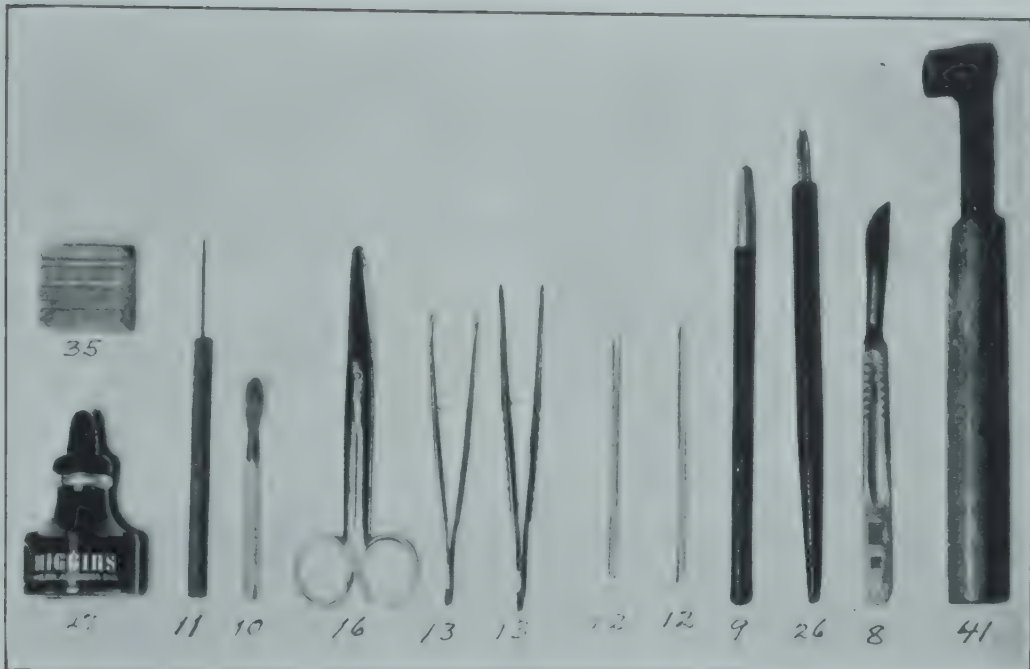


Fig. 12.—8, Scalpel to scrape off the frozen tissue from block holder. 9, Diamond pencil to scratch numbers on glass slides. 10, Camel's-hair brush to scrape paraffin sections from knife. 11, Teasing needle to pull and straighten frozen and paraffin sections. 12, Pointed glass rods to pull and straighten frozen sections. 13, Metal forceps to handle formol-fixed tissues and paraffin sections. 16, Scissors for cutting identifying paper numbers. 26, Pen to write identifying numbers on tissue paper with black India ink. 27, Higgins India ink for identifying numbers. 35, Vulcanized fiber for mounting celloidin blocks. 41, Hand wrench to open and close carbon dioxide tank.

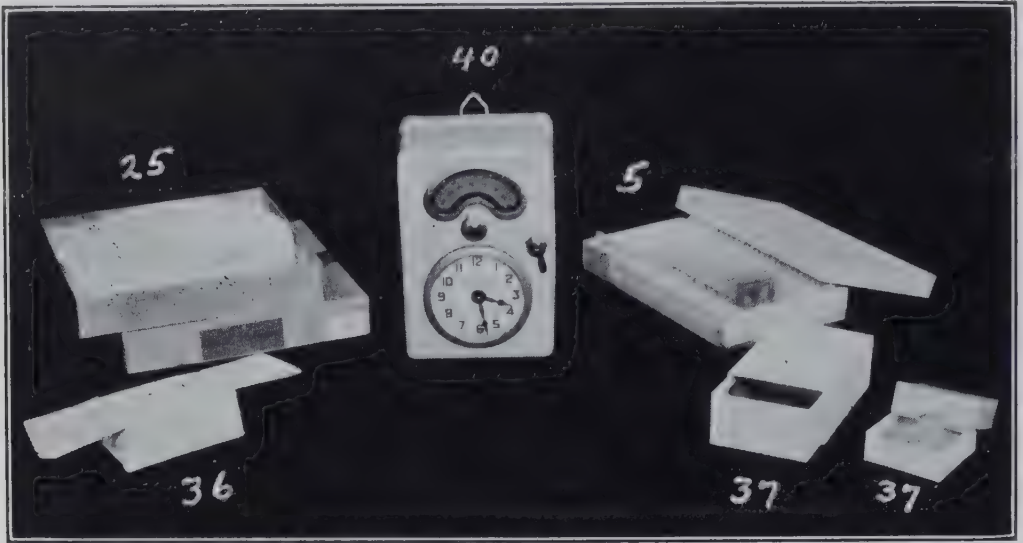


Fig. 13.—5, Wooden slide box, holding 25 slides. 25, Filterpaper (Whatman) for blotting sections. 36, Paper box for mounting paraffin blocks. 37, Large and small pillboxes for storing and keeping trimmed paraffin blocks. 40, Timer alarm clock to be used in timing the staining and fixing processes.

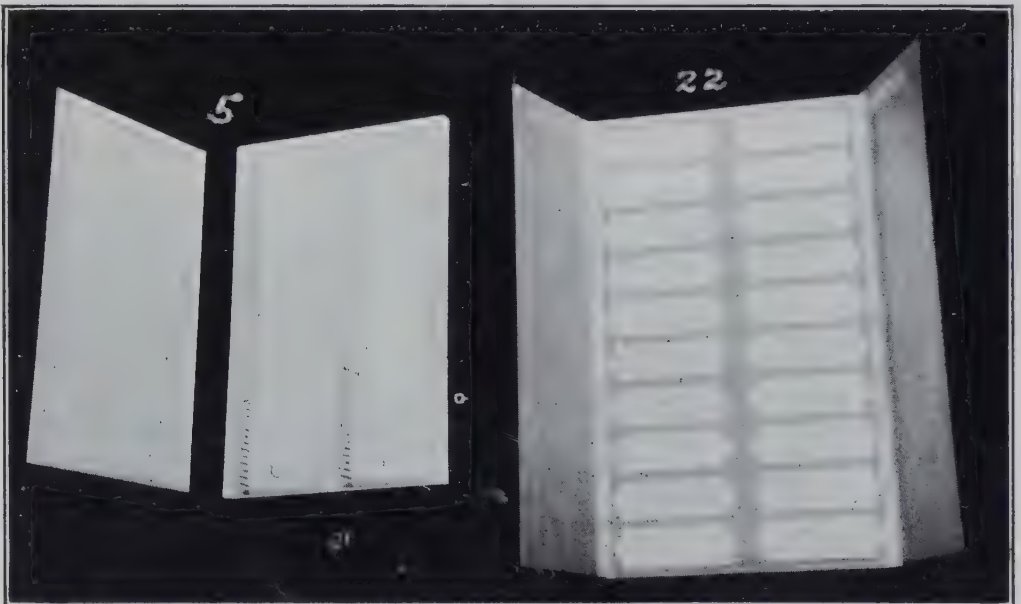


Fig. 14.—5, Wooden slide box, holding 100 slides. 22, Slide folders to keep freshly mounted microscopic slides.

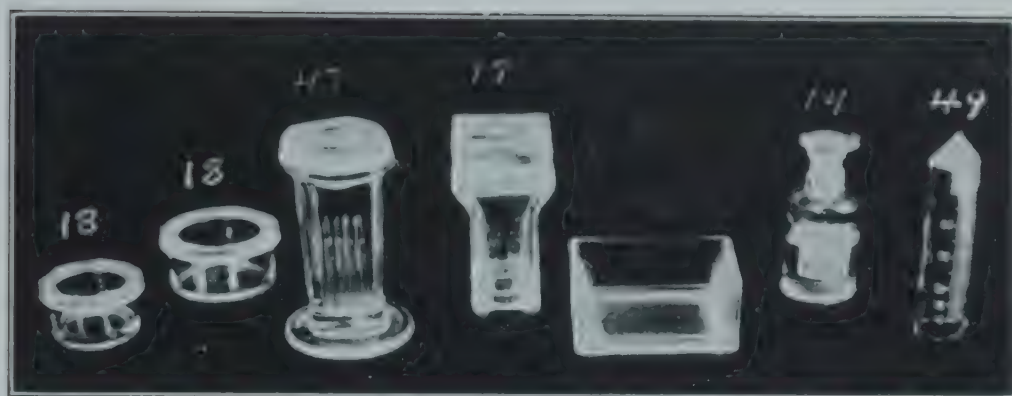


Fig. 15.—14, Drop bottle for storing oil and alcohol. 17, New type staining dish, holding 8 to 15 slides. 18, Stender dishes for staining loose sections, especially used in silver stains. 47, Coplin jar. 48, Large sized staining dish. 49, Graduated centrifuge tube (50 c.c. capacity) for concentrating body fluids.

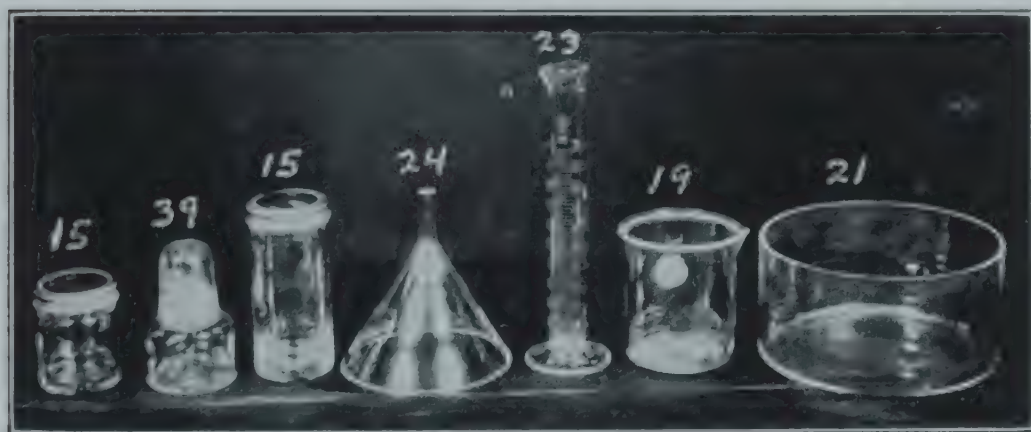


Fig. 16.—15, Bottles of various sizes for storing stains and solutions. 19, Beaker (200 c.c. size) to wash sections. 21, Water basin in which to float sections. 23, Graduate for measuring solutions and stains. 24, Funnel for filtering solutions and stains. 39, Narrow necked jars to hold gum dammar and immersion oil.

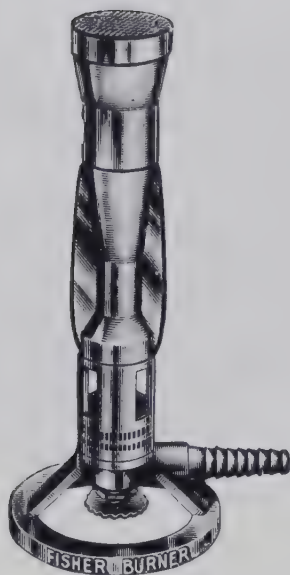


Fig. 17.—Fisher burner for heating paraffin block holder and heating solutions (Fisher). (Courtesy Braun Corporation, Los Angeles, Calif.)



Fig. 18.—Slide warming table for warming sections in various staining processes (Boekel). (Courtesy Braun Corporation, Los Angeles, Calif.)



Fig. 19.—Centrifuge to concentrate sediment of body fluids (International). (Courtesy Braun Corporation, Los Angeles, Calif.)



Fig. 20.—Electric oven for paraffin embedding (Precision). (Courtesy Braun Corporation, Los Angeles, Calif.)

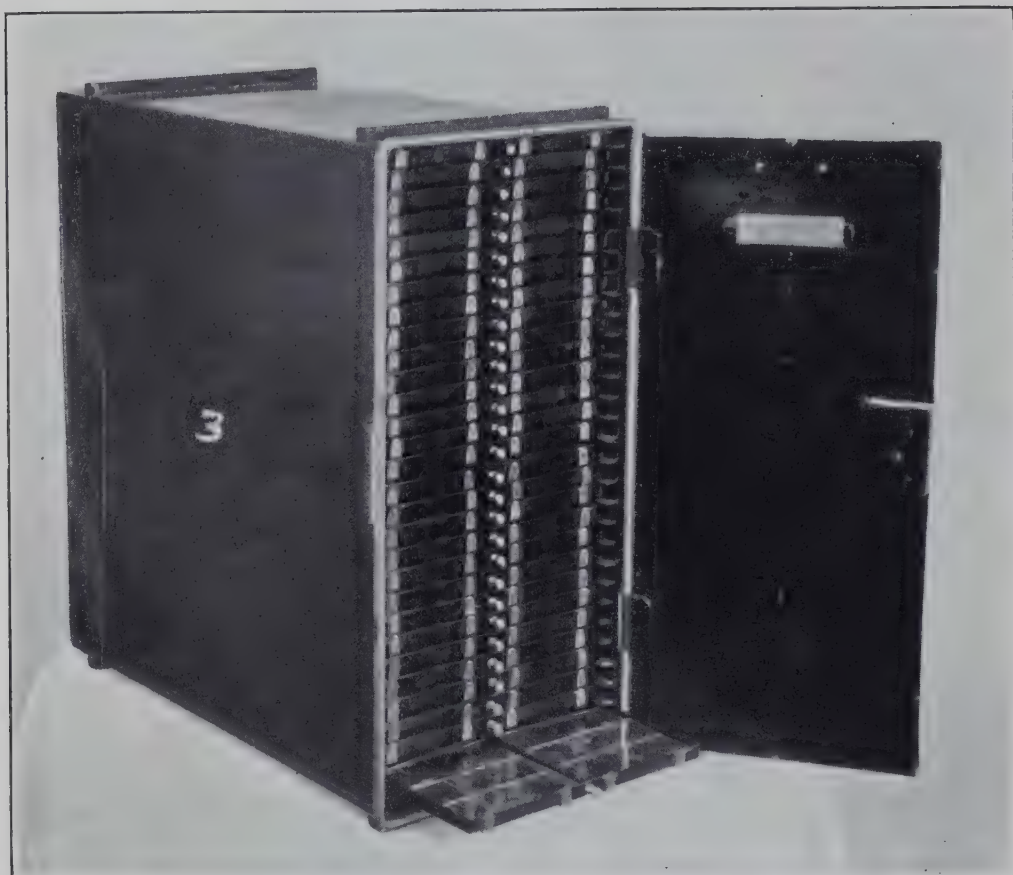


Fig. 21.—Small metal filing cabinet to store dry microscopic sections.

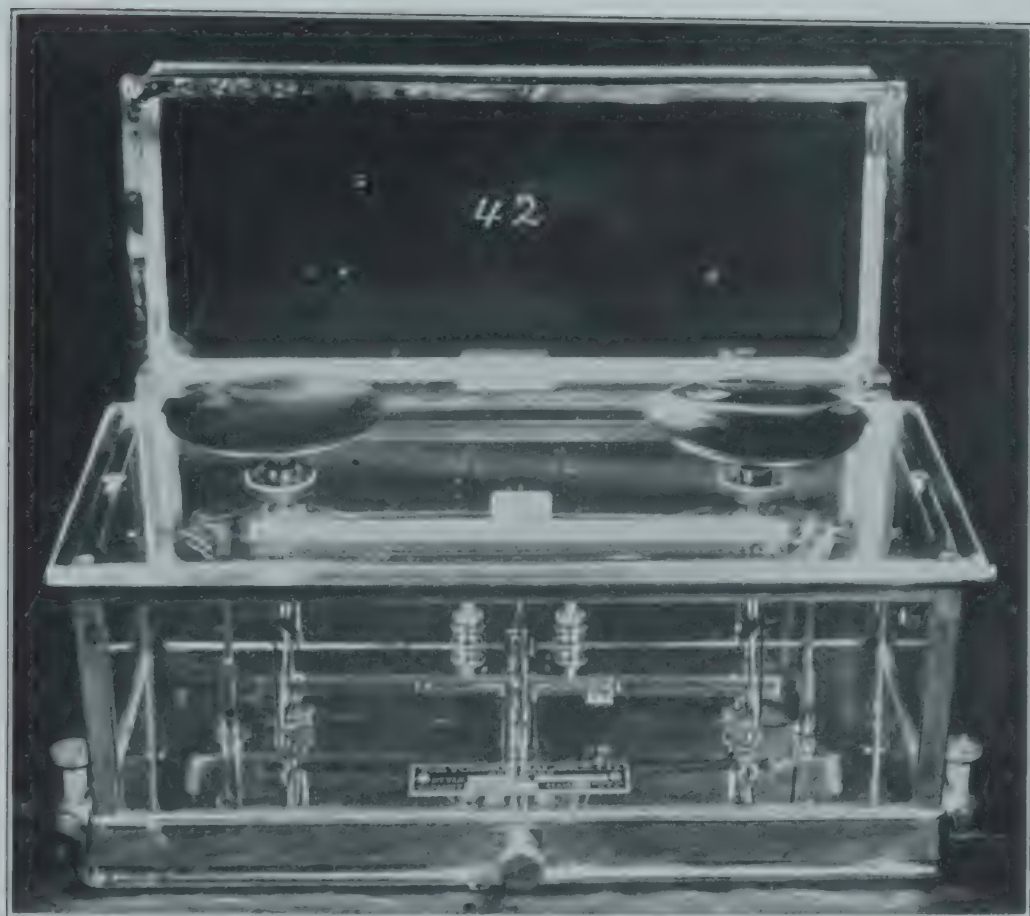


Fig. 22. Fine balances for weighing chemicals and reagents.

3. The plane-concave type, used for celloidin work with the sliding microtome.

For freezing and celloidin microtomes, standard size knives are supplied. For the paraffin microtome, it is better to have a larger knife, for after one position of the edge becomes dull, it will be necessary to move along the knife and bring a sharper edge into action. This will save the time and trouble of frequent honing and stropping. Razor blades have been found unsatisfactory.



Fig. 23.—Weights for balances (Becker). (Courtesy Braun Corporation, Los Angeles, Calif.)

Other Essential Equipment

Figs. 6 to 23 illustrate most of the items of equipment commonly employed and necessary in tissue laboratory work.

PART III

METHODS OF SECTION-MAKING

Standard Technics in Microsection

Tissues must be submitted to a standard method of section-making before they can be examined with a microscope. This involves the processes of dehydration, clearing, and embedding. The section-making methods are grouped under three headings:

Frozen Section Method ✓

Formol-fixed solid tissues are cut directly without embedding on a carbon-dioxide gas freezing microtome, but loose textured tissues are first subjected to an embedding process in gelatin agar or fixed in serum. The serumizing process has been found to be far superior to the gelatin agar method.

Paraffin Section Method ✓

Tissues fixed in any standard fixing reagent are first completely dehydrated, cleared, and then embedded in melted paraffin. Thin sections are cut on a standard paraffin microtome.

Celloidin Section Method

Tissues fixed in any standard fixing reagent are first completely dehydrated, cleared, and then embedded in a thick celloidin pool. Sections are cut on a celloidin microtome.

Method for Sectioning Body Fluids (Krajian Method)

When malignancy is suspected in chest or abdomen, with effusion into the serous cavities, fluid is aspirated and collected in large test tubes or flasks under sterile conditions and sent to the laboratory for microscopic examination.

The fluid is first poured into a 50 c.c. graduated test tube and centrifuged for 5 minutes. The supernatant fluid is then poured

off, and an amount of uncontaminated human or animal serum, equal to the sediment present, is added. This is shaken thoroughly and centrifuged again for 10 minutes. The resulting heavily packed sediment is placed in a paraffin oven at 56°C . for 1 to 2 hours, the time varying directly with the amount of sediment. The supernatant serum is decanted and about 5 c.c. of dioxane are added to cause complete coagulation of the mass, which takes place within 2 to 3 hours. It is then fixed in 10 per cent cold formaldehyde for 24 hours, or in hot formaldehyde in paraffin oven at 56°C . for one hour, after which it is ready for sectioning by one of the standard methods. We have used the frozen sections on all of these sediments, but equally good results can be obtained with paraffin or celloidin.

For staining, clearing, and mounting, follow the staining technic described under "Routine Staining Methods."

Generally, these sections form air bubbles which can be eliminated by the following procedure: Stain sections in usual manner and carry to carbolxylol. Blot thoroughly in fine filter paper, dip in thin celloidin, and place in carbolxylol. Repeat process once and blot again before transferring to xylol.

Frozen Section Method for Preparation of Permanent Tissue Sections

(Krajian)

For the preparation of microscopic sections there are three methods in general use. Of these, the paraffin method is the most popular, while the other two, the frozen and the celloidin procedures, are not extensively used.

The celloidin method is not practical for routine work because it is time-consuming and expensive. However, it is valuable in preparing sections of tissue of hard texture, such as eyes, bones, and cartilage.

The much neglected frozen section method is a very convenient way of preparing routine microscopic sections. Its advantages are rapidity, economy, preservation of cellular structure, and absence of shrinkage. The limited use of such a desirable method is due in part to certain difficulties in the technic. These difficulties are easily overcome when certain details are understood.



Fig. 24.—Equipment and reagents for staining frozen sections, method of A. A. Krajian.

The Use and Care of Microtome Knives

Lorimer Rutty, B.Sc., Ph.D.*

Since all successful practice is based on sound theory, a knowledge of the theory of cutting is essential to the intelligent use of cutting edges.

A theoretically perfect edge may be described in very simple terms. It is merely the junction of two smooth plane surfaces. To appreciate the connotations of that simple definition, let us consider the action of cutting.

Cutting action, as applied to the cutting of sections, is essentially that of parting or dividing. The knife enters the specimen to be cut and divides it into two parts, the parent body and the slice removed.

No action takes place without an equal and opposite reaction. The block and specimen so cut must withstand the effort of parting and, for minimum distortion of the specimen, the effort involved must be held to a minimum.

To achieve maximum penetration with minimum effort implies not only that the two planes shall meet at a point of zero cross-section, but that the angle so formed shall be as small as possible, in order that the fibers of the specimen penetrated shall be subjected to the least displacement at the instant of parting.

It becomes apparent that in describing a theoretically perfect knife edge we are also describing a theoretically perfect wedge.

The angle of this wedge is important. Although an acute angle is desirable, it is not always practical.

While the cutting or parting action takes place at the point, the effort of so doing is reflected back into the bevel, diminishing in intensity in proportion to the increase in cross-section.

An apt simile would be that of one man standing on the shoulders of two or more men in order to push up an overhead trap door. Just as the effort of the man on top is reflected downward and must be supported by the men below in proportion to their numbers, so the bevel or shoulder of the blade must support the cutting edge.

From the standpoint of strength and support, a 90 degree bevel would be ideal, since the more rapid the increase in cross-section, the more rapid the dissipation of energy.

*The authors are much indebted to Dr. Lorimer Rutty, of the Micro-Sharp Company, Box 363, Niagara Falls, N. Y., for this original contribution.

Something along these lines is used for cold chisels, but, for the reasons outlined above, would be impractical in the microtome.

The optimum angle for microtome knives seems to be about 14 degrees. This angle represents the maximum penetration and, hence, the minimum parting effort that can be achieved, with adequate support for the cutting edge under normal circumstances.

Our definition of a theoretically perfect cutting edge for microtome knives now resolves itself into two smooth plane surfaces meeting at an angle of 14 degrees.

Practically everything that modern science has learned about the correct method of sharpening knives is directly opposed to the popular theory and so it will be in order to start with the finished product and work backward, or from effect to cause.

In Fig. 25, we see an unretouched photograph of a cutting edge, magnified about 1,700 times, as printed. This knife was not cleaned before photographing and a lot of the marks are air-borne debris attracted to the knife by static electricity as is the case in Fig. 57.

This comes close to satisfying our definition of a theoretically perfect cutting edge, the junction of two smooth plane surfaces, since it is obvious that the blade is flat right to the edge.

Perfect linearity of the edge is a function of the smoothness of the surface, as explained later in dealing with honing. While the surface shown is not perfectly smooth, the imperfections, at this magnification, are too small to disturb seriously the linearity of the edge.

A flat, wedge-shaped edge will part the specimen to be cut at a uniform rate of speed, causing a minimum of distortion and displacement of the material in the section, but the accomplishment of a perfect cutting edge is not alone a matter of skill and experience.

The quality and "state" of the steel, which may be loosely defined as the size and shape of the grain structure, owing either to its composition and manner of hardening in its manufacture or to the use or abuse it has received since, are equally important and have an influence on the results obtained.

It may be in order to mention a few of the things which make a perfect edge difficult to obtain and some which make it impossible.

It is not good enough merely to cut a knife to a 14 degree bevel and then proceed to strop the edge until it is rounded to a final angle of 30 to 50 degrees in order to achieve linearity.

Such a steep, abrupt edge increases the cutting effort involved, leading to tearing, distortion, and excessive compression of the section. This added effort has to be borne by the knife edge.

Fig. 25.

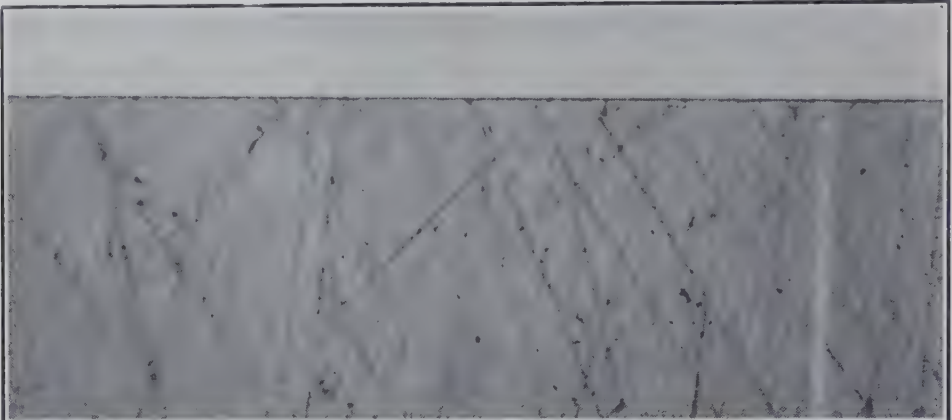


Fig. 26.

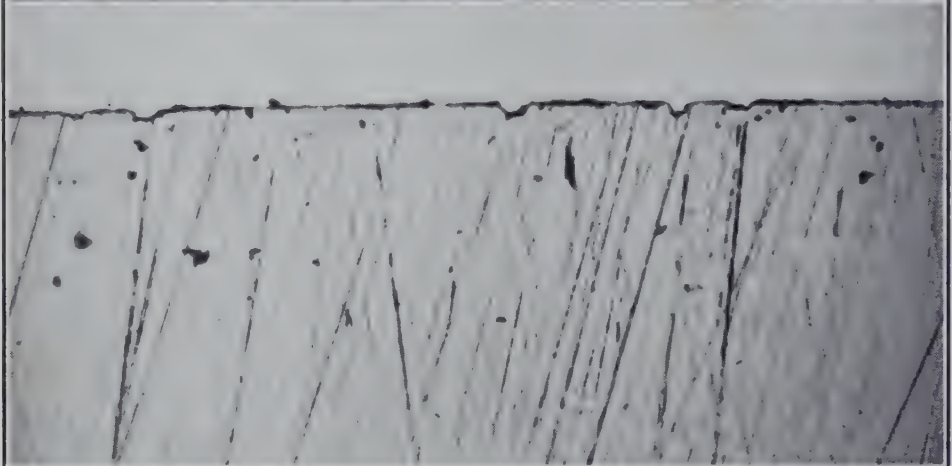
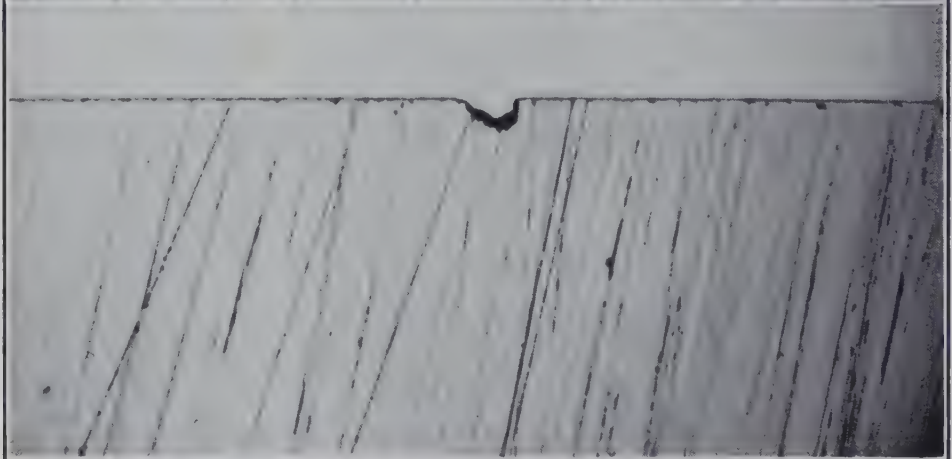


Fig. 27.



In addition to the above, it is common practice among technicians to operate their microtomes at excessive rates of speed. Time is the essence of all work performed. If a section is cut in half the milli-

seconds normally required, the effort must be at least doubled and this, too, increases the load borne by the knife.

Another habit, common to most technicians, is to do most of their cutting in the center of the knife, causing a disproportionate amount of wear in this area.

Fig. 26 shows a condition that is typical of a knife that has been used too much in the center. "Work-hardening" is a condition that is familiar to all who use steel to cut, punch, or form. After a customary term of service, a die or punch is removed and stress-relieved by heat treatment.

Theoretically, sharpening a knife should obviate this procedure if the whole blade were used evenly until dulled and the work-hardened surface removed on the stones.

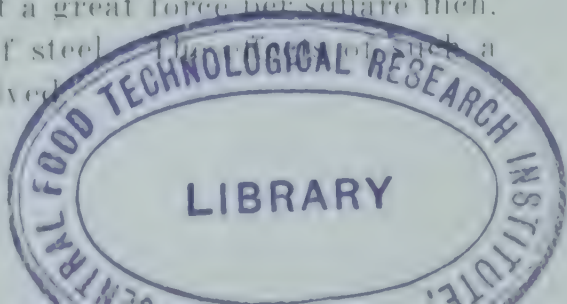
In practice, however, the observation of many thousand blades shows that the technician tends to concentrate the work in the center area, with the result that the effects of work-hardening go deeper than may be justifiably cut away in any one sharpening without sacrificing the potential usefulness of the balance of the blade.

Failure to clean the blade after use can have serious effects if the matter is corrosive. It might be interesting to note in passing that blood seems to be the most corrosive of all common substances when in contact with high carbon steel. A spot of blood left on an eye knife, for instance, will eat its way into and under the surface, enlarging the hole as it goes, until a comparatively tremendous cavity is formed beneath the surface.

While this is serious enough on large cataract knives and keratomes, on small cataracts (Wheelers and Zieglers) the relative proportions of the cavity may be so great as to necessitate the knife being seriously distorted in shape or discarded altogether.

In Fig. 27 we see an example of a corroded cavity in an otherwise carefully handled knife. The corroded area beneath the surface is far greater than that on top and here again the entire blade must be sacrificed for one area.

Careless handling of knives has far greater consequences than is commonly known. A comparatively light blow on an area of a very small cross section can represent a great force per square inch, easily beyond the impact strength of steel. The effect of such a blow extend far beyond the area involved.



A pair of very rare and unusual pictures, Figs. 28 and 29, show a blow from two different directions.

In Fig. 28, the knife was leaning at an excessive angle in the microtome and encountered some metallic object traveling relatively downward. The metal has been distended outward like a scoop, far out of focus, and the base of the area is badly distorted.

Fig. 28.

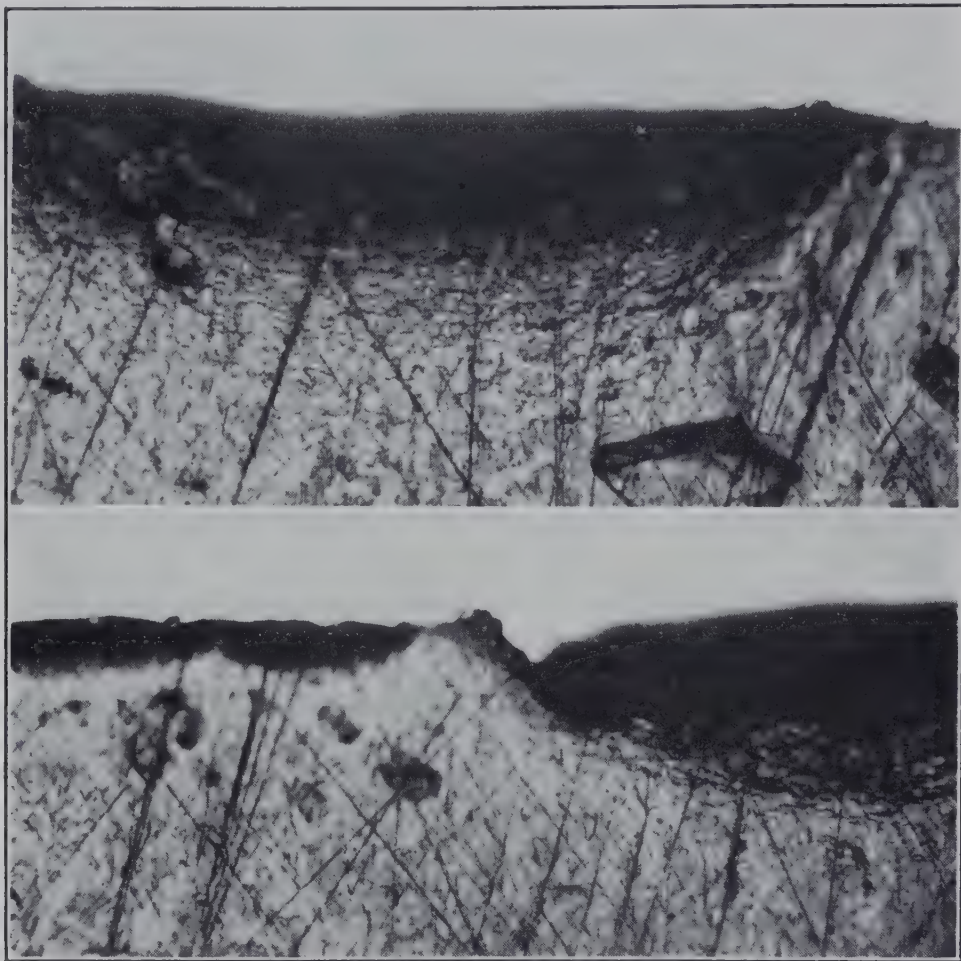


Fig. 29.

In Fig. 29, the blow was normal to the axis of the knife. Tracing the line of focus indicates that the metal has been compressed downward and outward into a pocket and stretched above the adjacent plane of the bevel.

In both these instances, the area of deformation of the grain structure extends far behind the involved area and the section will break away during or before polishing, as in Fig. 26.

Some knives are not susceptible of being sharpened. A practical example of this occurred in the making of dermatome knives for skin grafting. A number of blades were produced with the intention of their being so hard that, once sharpened, the edge would last almost indefinitely.

Fig. 30.



Fig. 31.

The result was the opposite to that intended. The effect, for all practical purposes, was as if each grain had become so large that its bonding to adjacent grains would not sustain the action of cutting and polishing but was wrenched loose. Even the blades on which it was possible, at great effort, to produce a passable edge, would not give satisfactory service but chipped and broke at the edge. These blades were later discarded and others of more suitable steel were substituted.

Another interesting and instructive example is seen in Figs. 30 and 31. Fig. 30 is the point of a cataract knife, brand new and ready for use. Fig. 31 is the same knife, properly sharpened and magnified 750 times. Only fine-grained steel is susceptible of being so sharpened. With coarse-grained steel, the whole tip will break away and the point must be so proportioned that its final angle may have to be almost 90 degrees in order that only a small part of each grain shall be exposed.

Many knives in current use do not justify their being kept in service. Most of these are the very old knives which were made either to an outmoded concept of proper hardening or subsequently have been used beyond their capacity to sustain effort without changing their character.

It is noted with regret that a lot of comparatively new knives seem headed for early failure from the observed effects of overwork, and the blame will be ascribed to the manufacturer. A suggestion is made that a service be provided whereby knives, after a term of use or upon showing evidence of the necessity, could be so heat-treated as to restore their original structure.

No facts are available as to the practicability of this service. The necessity has not hitherto been recognized and therefore it is not known that any such service has been attempted.

With the continuous enlargement of public health service and the increasing emphasis on speed in the laboratory, manufacturers may be so directed in self-defense.

Fig. 32, although a rare photograph, depicts a condition that is not rare, but frequent, among older knives. Sections of the steel break away, one might almost say spontaneously, as if cutting away of the adjacent area released some pent-up internal stress. Further cutting serves only to alter the location without reducing the incidence of the broken areas.

Continued exploration could become a Pyrrhic victory, since the ultimate answer might coincide with the termination of the useful life of the blade.

In the center of the picture, a section has sprung upward, arching above the plane of the bevel although still restrained at the ends. At the right, a section has turned so that its reflected light is no longer normal to the microscope tube. Toward the left, a piece has gone and, at the extreme left, partly obscured in the printing, two pieces have joined forces and left en masse.

It is not possible to treat the functions of honing and stropping separately since they are inseparable, the latter being merely a further stage of the former.

The sharpening of microtome blades in the laboratory has not proved to be an unqualified success if the observation of many thousand knives can be considered representative. The routine maintenance, by stropping, of a properly sharpened knife is, or can be, made an easy task requiring less than one-tenth of the energy customarily dissipated by faulty technic.

As to honing, competent medical technologists are not necessarily good mechanics and might have far less aptitude for medical work if they were.

Nevertheless, many technicians attempt to sharpen their own knives on the strength of a few instructions imperfectly learned from others who are no more qualified to teach.

Not only is the popular theory of microtome knife sharpening heir to the confusion of barbershop practice, but the technician is provided only with barbershop tools. While there may be exceptions that have thus far escaped notice, hones and strops currently sold to technicians are primarily barbers' tools or a glorified version thereof.

A hone of proper size for laboratory use would have the same dimensions as a proper strop and for the same reasons, as will be apparent later.

A knife should be honed in register with the back with which it will later be stropped and stropping should be done only with the back to which the knife was cut. Many thousands of hours are wasted in laboratories throughout the country in stropping knives that have been cut to an unknown angle.

Generally, to save time, the knife is cut to a short, steep bevel, an angle far greater than that implied by the back. Resort must then be had to the barbershop practice of using a sagging strop in order to reach the cutting edge.

After a half hour's hard work, which is at best sheer guesswork, the technician probably has achieved an edge rounded to an excessive angle and close to the end of its useful life before being used.

No hard and fast rules can be laid down for honing, since there would be as many exceptions as there are knives and will be dependent largely on the use and care the knife has received.

Fig. 32.

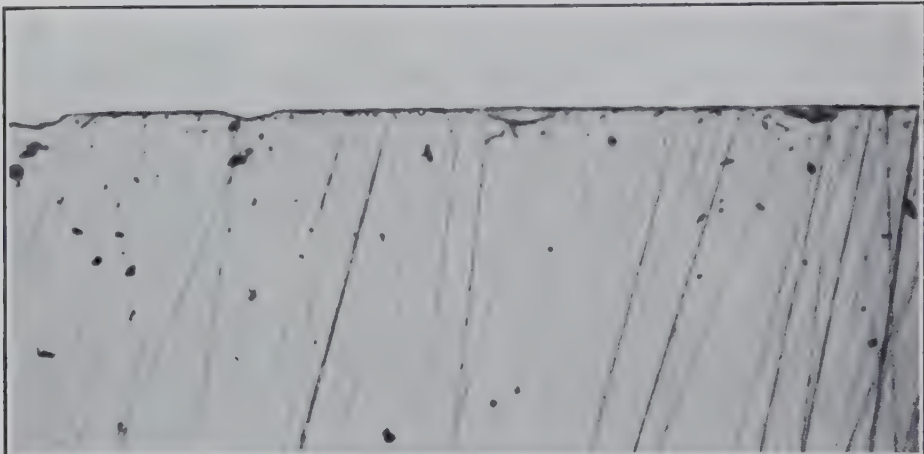
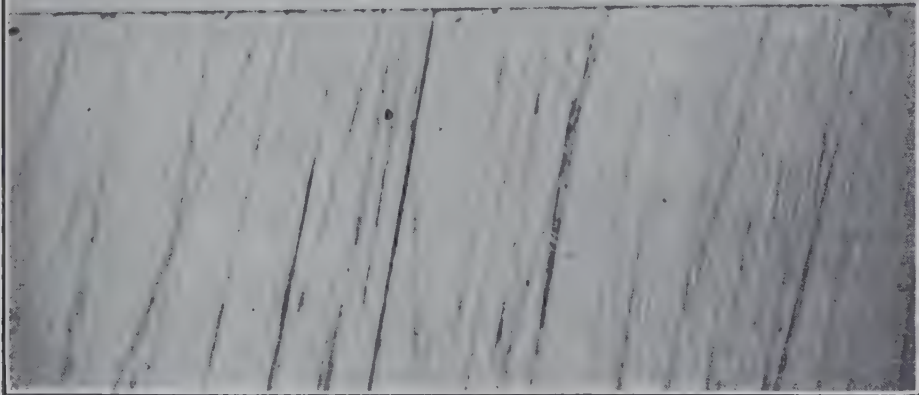


Fig. 33.



Fig. 34.



Two points are worthy of note, however, as applying to all hones. The first is never, under any circumstances, allow oil, mineral or otherwise, to be put on the hone. The purpose of using any liquid on a hone is about 10 per cent for lubrication and 90 per cent to remove the cuttings of metal as they accumulate. Any viscous liquid placed on the hone tends to form the metal cuttings into a paste, with the ultimate results shown in Fig. 33.

The liquid used on the hone must flow rapidly enough that chips are removed between strokes and a clean hone is presented each time. The ideal liquid for this job is a form of water-white kerosene. To try to use flowing kerosene in a laboratory would be impractical. The only alternative, then, is plain water.

Honing should be done on a board placed over a sink and an adequate stream of water from a rubber hose allowed to flush the hone continuously. With care and the use of successively finer hones of the right type for the knives in question, results may be obtained similar to Fig. 34. This shows a knife cut, ready for polishing, magnified about 1,700 times as printed.

Second, clean the hone before and during use. The black film that a hone develops from the metal it is cutting should be brushed out with a good quality nailbrush under running water, using soap if necessary, until the hone is clean. If your hone has been oiled and you cannot boil out the oil with a detergent, its replacement is advisable.

The amount of stropping that will be required by a knife that has been freshly cut and is in exact register with its back, as in Fig. 34, will be subject to too many variables to allow of a generalization, but it will be infinitely less than under the accepted hit-and-miss method, and some idea may be gained from a study of a used knife.

Let us assume that we have been using a sharp knife that was cut to a plane surface in register with its own back. We will have cut some 75 to 250 blocks before stropping, depending on the nature of the sections, but now the knife is becoming dull and needs stropping.

The back must fit tightly enough that it does not slip with each stroke. Backs made of spring steel are useless for either honing or stropping, as they cannot be tightened enough to prevent slipping by any means available to the technician, and most of those

observed have been badly warped in the tempering, making accurate register impossible.

Backs should be made of ordinary seamless tubing, the natural spring of which is adequate for the purpose. They should be regarded as expendable and those showing excessive wear or hollowed in the center should be discarded.

To tighten a back, lay it on the edge of a projecting table top as in Fig. 35, and squeeze as if to crack a walnut. A back made of 22 gauge tubing may be tightened by almost any man's hand.

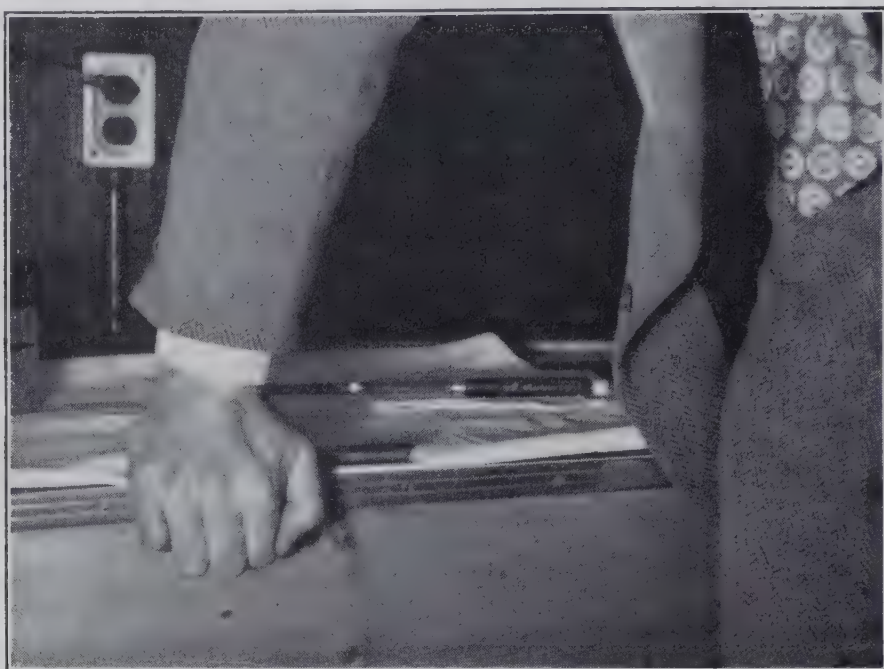


Fig. 35.

Tighten one-half of the back at a time and try it before proceeding further. It is easier to tighten a back than to loosen it. Although the latter is done with a wedge in the same manner, a suitable wedge 7 inches long would not be found in many laboratories.

Many technicians insert the knife about an inch into the back and then give a vigorous downward lunge against the bench. What happens in many cases is that the back and blade bend out of line, the wider part of the knife is jammed into the opening of the back, forcing the opening out of round and out of shape and altering the whole register between back and bevel.

The back should be held as in Fig. 36, and the blade gently rotated from side to side as it is forced on:

Backs that apparently are too tight will go on with comparative ease if a little guile is used rather than brute force.

Lay the knife on the table resting on its back, the edge upward, and, holding the blade (not the handle) at both ends, tap the back on the table to relieve any strains and settle the back in its place.

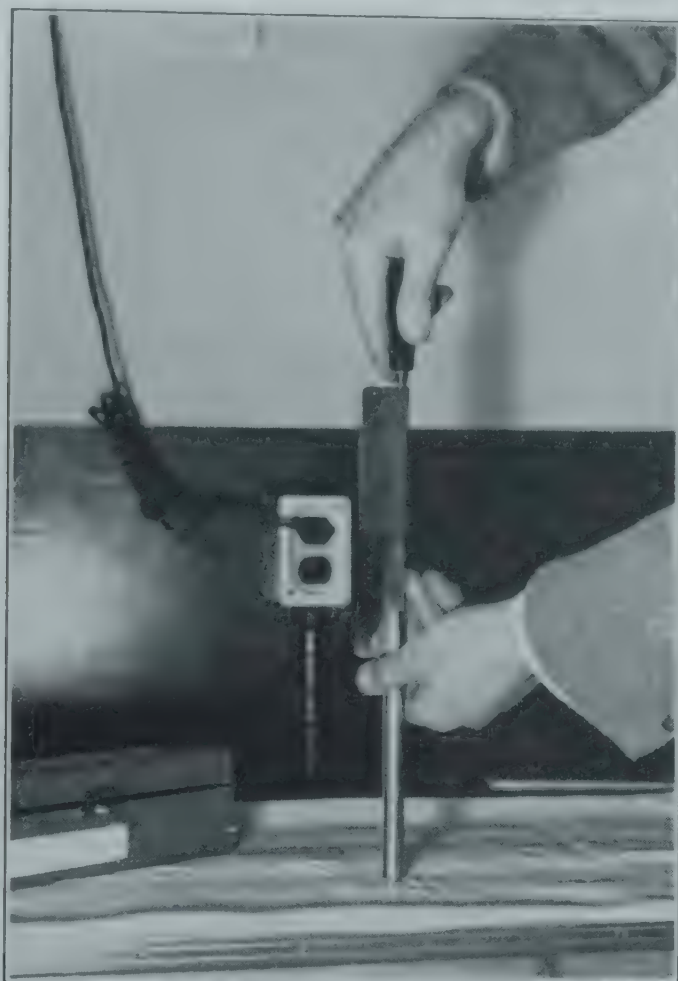


Fig. 36.

Every laboratory should have a piece of plate glass about 6 × 12 inches on which to test the knife to see if the four corners of knife and back are resting level. If not, twist the back at alternate ends to see which end is at fault. Try both sides of the knife. It is important that both ends of both sides of the knife should make contact with the glass plate.

Removing a tight back is very simple if gone about in the right way. Buy a small vise and mount it at the end of the bench, the jaws at right angles to the face of the bench. Grip the end of

the handle in the vise and arrange the hands as in Fig. 37 (largely to know that all your fingers are accounted for). A sharp tug will loosen almost any back.

Do not pull the back all the way off or the blade will swing downward in the vise and may fall. If pulled about three-quarters off, the balance of the back will come off easily in the hands after removal from the vise.

Always clean the knife before stropping, even if you are not finished using it. Wax must not be allowed to get on the strop. With a suitable applicator, flush the blade with xylol. Fold a piece of soft cleaning tissue to present a flat surface, hold in the left hand (for right-handed persons) and draw the blade across the tissue.



Fig. 37.

Always wipe the knife on the paper; never wipe the paper on the knife. Repeat the operation with fresh xylol and a fresh surface. One wiping will not remove all the wax.

Now that we are ready to strop the knife, we find, as outlined before, that no strop is provided for this purpose. All we have is a barbers' strop as in Fig. 38, which we have had cemented to a soft pine board, since one stroke of a properly cut knife on a sagging strop will completely ruin the edge by rounding it and all further stropping will be pure guesswork.

To strop a 7 inch knife on a 3 inch strop is virtually impossible under normal laboratory conditions. It is inevitable that a hone or strop narrower than the knife shall cause more wear in the middle area than at the ends and the blade quickly becomes bowed in the center as in Fig. 39.

The left hand should be on the knife at all times, with the tips of the fingers resting halfway between the back and the bevel, and the pressure should be governed entirely by the left hand, the right hand merely providing the movement.

Fig. 38.

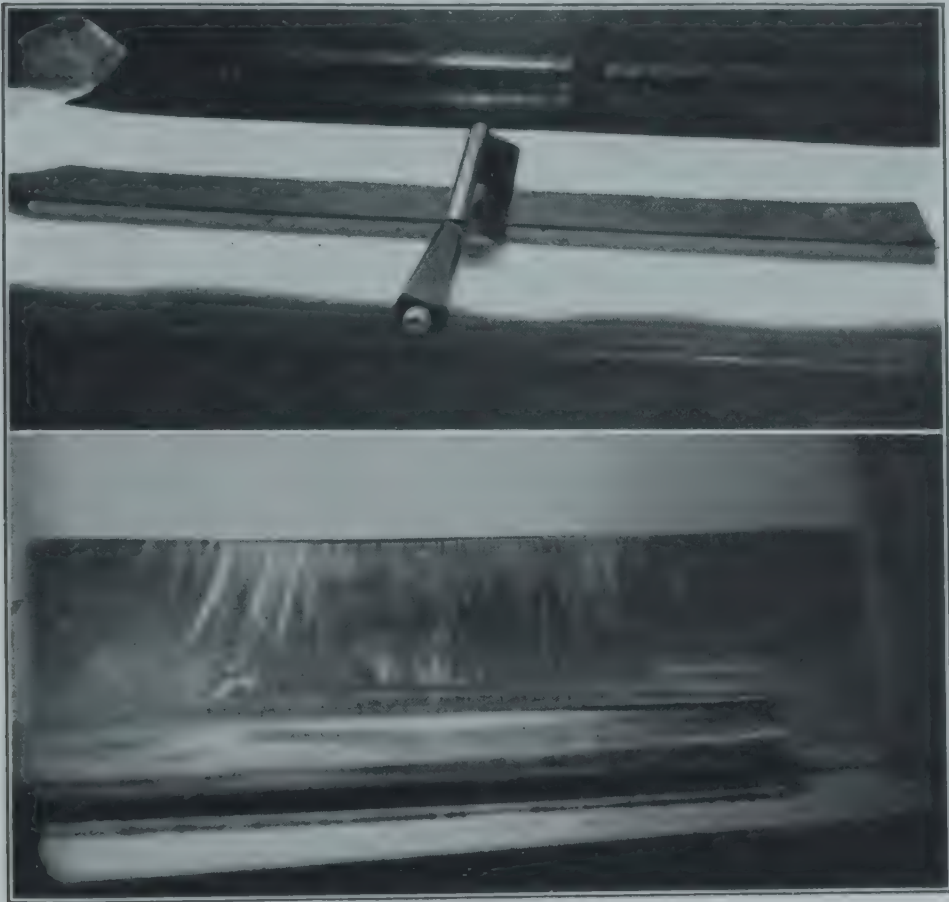


Fig. 39.

In addition to controlling the pressure, it is vital that the left hand should keep the knife level on a narrow strop and counteract the tendency of the right hand to droop or lift. If the knife is not balanced on the strop and is allowed to tilt, gouging of the edge on the side of the strop occurs as in Figs. 40 and 41. In Fig. 40, the strop was in about normal condition, but in Fig. 41 the strop was dry and brittle and cut into the steel almost like a hone.

A suitable strop for laboratory use would be 12 inches square for laboratories that use up to 10 inch knives, and 9 inches square for those using only 4½ inch knives. This latter size seems to represent about 95 per cent of the knives in use today. No cowhide strop has ever been observed that was suitable for laboratory use.

Fig. 40.

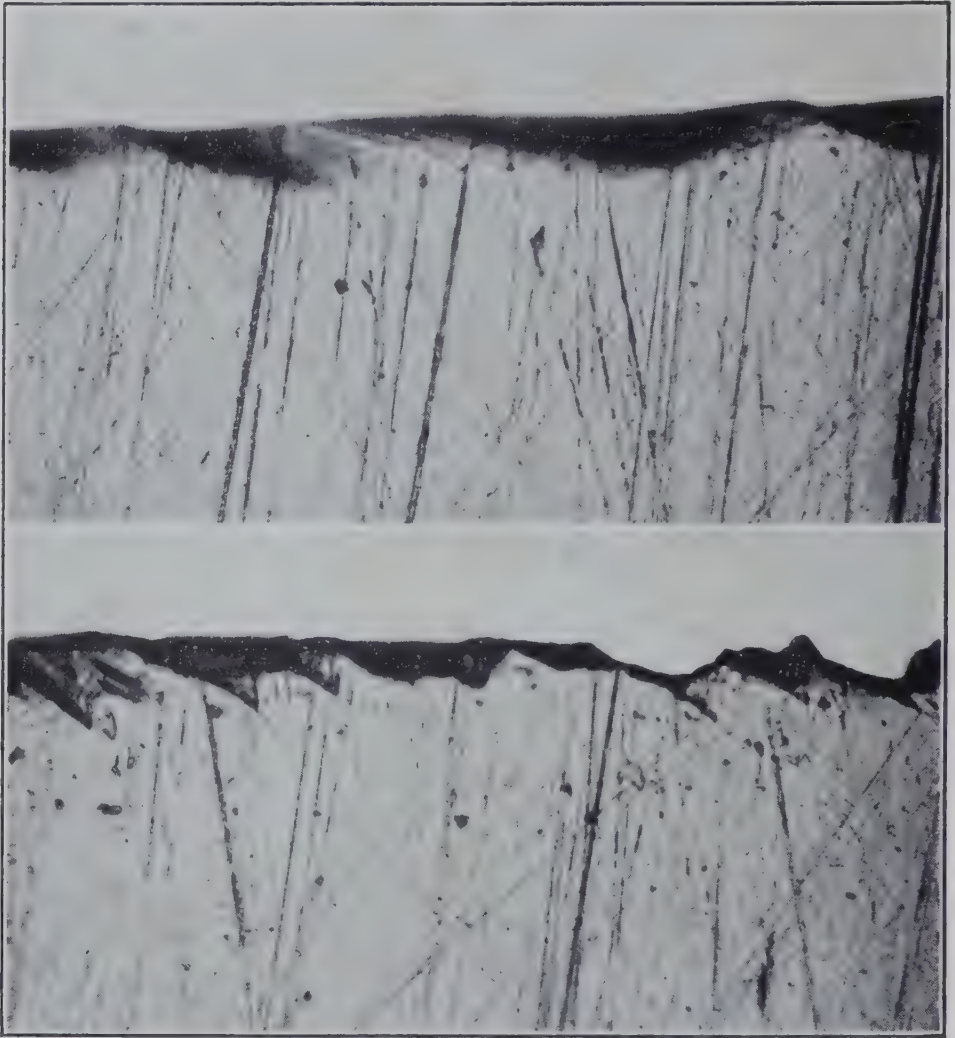


Fig. 41.

The strop would be made of horsehide, the part known as horsebutt, taken from the hindquarters of the animal between the backbone and the "equator," where a sharply defined line of wrinkling occurs. The wrinkled part is of no value for a strop.

The finish of the strop would be that variously known as Scotch Shell, Pony Shell, etc., and has a polish almost equal to the knives themselves, as may be observed in Fig. 42.

This is a 12 inch square strop, suitable for knives up to 10 inches. Although no 10 inch knife was available at the time for inclusion in the photograph.

FIG. 42.

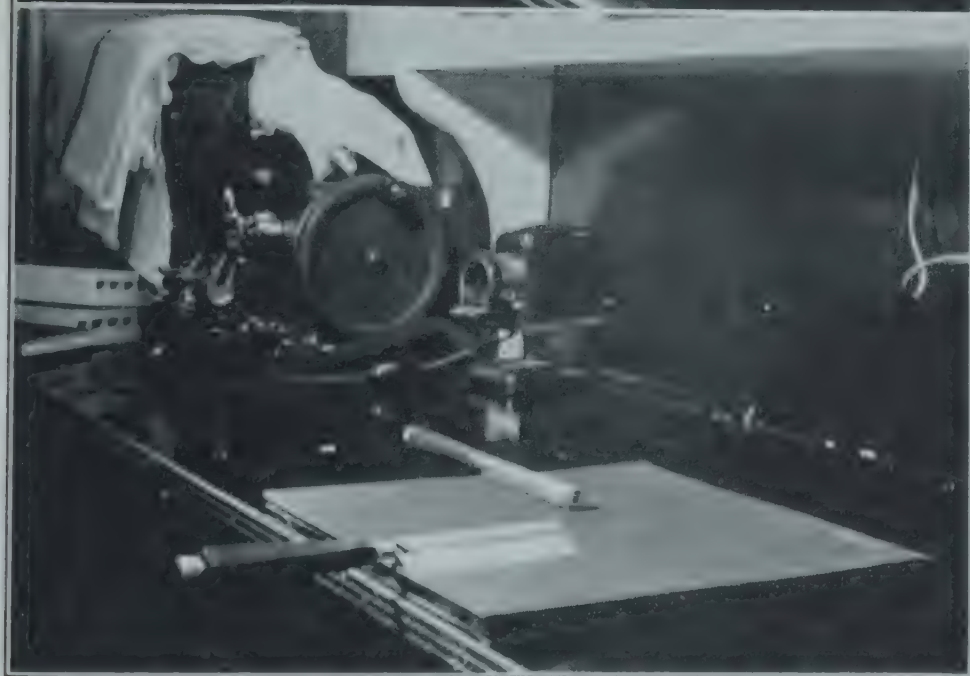
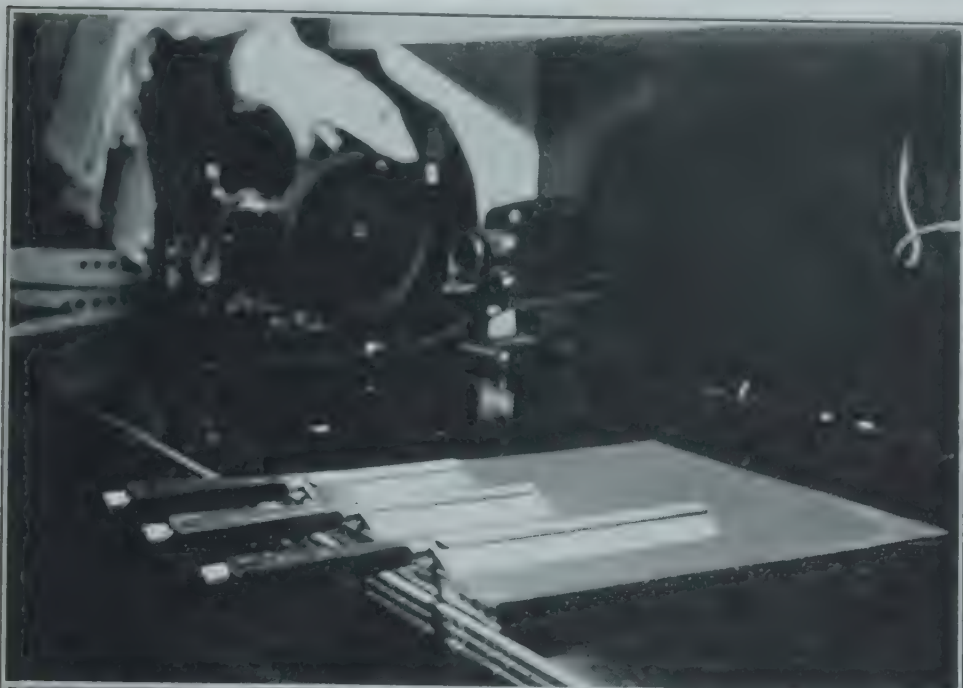


FIG. 43.

Observe in the near right-hand corner of the strop, the line of wrinkling extending diagonally. This line on the animal was horizontal at the "equator" of its girth. The near right-hand corner

of the strop pointed downward and the far left corner upward. The value of this strop is minimized by this wrinkled area, but, as it is probably the only such strop in existence, it cannot be helped.

To use this Scotch Shell strop, wipe it lightly to remove any possible dust and then buff vigorously with a ball of bath towelling held in the hand. Now, with the palm of the hand, rub the leather until both hand and strop are warm, following this with another buffing.

This procedure is adequate to keep this kind of leather in good condition for years, the leather growing more smooth and lustrous the more it is handled and buffed. The strop shown has been used for odd jobs for some seven years, with the care outlined above, and is in perfect condition.

Never allow mineral oil to come in contact with a strop. One drop of mineral oil will spoil the polish of that area for all time and is another reason why oil should not be used on a hone, since some must inevitably get on the strop.

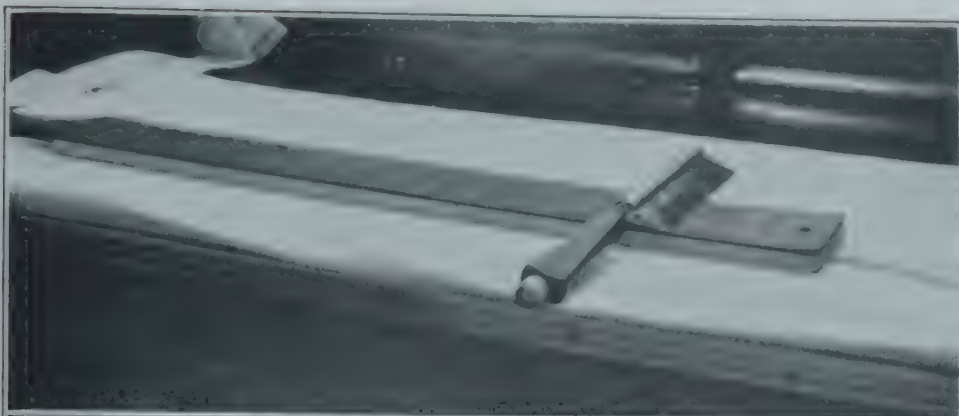
Leather that is dry may be restored by using a 10 per cent solution of sulfonated castor oil in ordinary tap water. Pour some in a saucer and, with the tips of the fingers, moisten a very small area; follow immediately with a vigorous buffing to a high polish before proceeding to the next area. The trick is not to let the leather soak as this will bring up the grain and make the finish rough. Do not try to restore full moisture in one application, but repeat at intervals of two or more days. If a solution much stronger than 10 per cent is used or too many applications are made, it tends to make the leather gummy.

If leather has become excessively dry and brittle as was the case in Fig. 41, which was cowhide sole leather to begin with, the time involved does not justify its reclamation and it should be replaced.

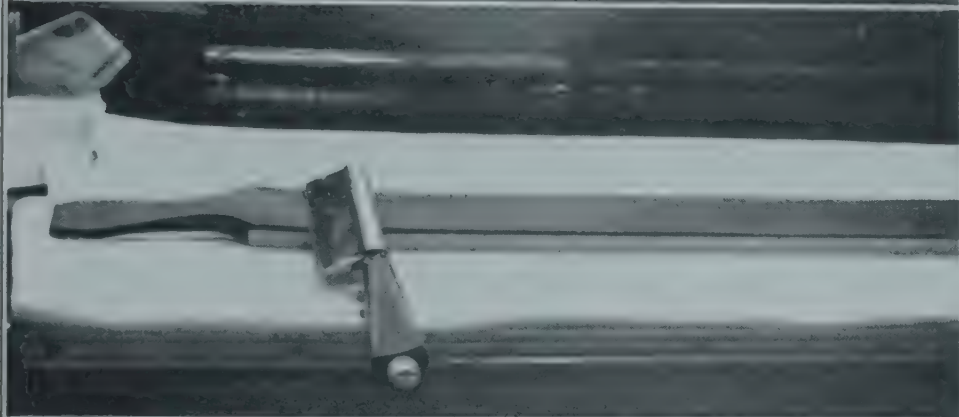
A strop should be used on all four sides as shown in Fig. 43. If one side becomes scratched by a nick in the knife, three other sides are available with the one buffing and rubbing and the scratch may be polished out later.

Although we have shown and described a strop suitable for laboratory use, no such strop is commercially available at the present time. Consequently, we must do the best we can with a barber's strop until one is available.

44.



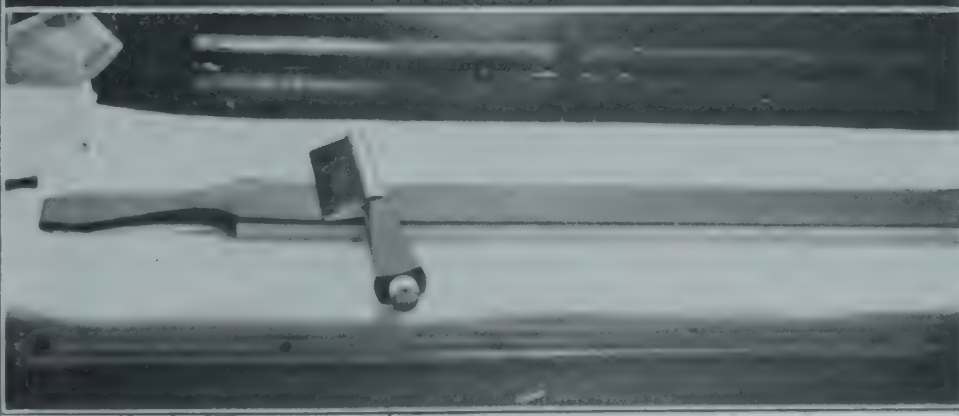
45.



46.



47.



Assuming that the back and blade are in register and that the bevel is a plane surface, we may accomplish as much in fifteen or twenty seconds as in a half hour of hard work when using guess-work.

The successive steps are outlined in Figs. 44-47.

Place the knife on the strop as in Fig. 44, the heel on the strop and the toe overhanging. Place the left hand on the blade to balance it and prevent gouging as in Figs. 40 and 41. Draw the blade diagonally from top left to bottom right and roll it over on its back as in Fig. 45, ready for the return stroke along the same diagonal path from bottom right to top left.

Now move the blade over as in Fig. 46, the toe on the strop and the heel overhanging, and draw the knife diagonally from top right to bottom left. Again roll the knife over on its back and return from bottom left to top right.

These four strokes constitute one cycle and three such cycles should prove adequate for the first stropping, after a term of cutting, of a properly sharpened knife. Try the knife before proceeding further. Most knives are overstropped.

Pressure during the first stropping should be quite light; the natural compressibility of the leather is what actually does the work. Successive stroppings will require a gradually increasing pressure as the bevel tends to lose its plane character and becomes rounded at the edge.

For the sake of clarity, the knife shown in Figs. 44 to 47 has been depicted as traveling from one end of the strop to the other. This length of travel is neither necessary nor desirable. The knife merely should travel far enough to bring the overhanging end a little more than on the strop at a direction of travel of something less than 45 degrees.

The actual travel along the strop should not be more than four or five inches for a 4½ inch knife. Longer knives will require a longer travel but the net stropping action per inch of blade will be the same. This length of travel may seem revolutionary to some technicians but, on a proper strop as in Figs. 42 and 43, the travel should not greatly exceed two inches, with correspondingly more cycles. The essence of successful stropping is a succession of short, light strokes with alternate sides of the blade.

Apparently, most technicians tend to press harder on the going away stroke than on the return, because thousands of knives have

been so observed. The operator should stand so balanced that each direction of stroke shall have exactly the same pressure. Either too long a stroke or uneven pressure will produce a wire edge as in Fig. 48. Fig. 48 happens to be a dermatome blade used in skin grafting, but was the only photograph available at the time. It is virtually impossible to make long strokes uniform in their stropping action and the net effect will be that of uneven pressure.

Speed in stropping is to be avoided. One full second should be allowed for each stroke; 12 to 15 seconds for three cycles of four strokes each. For each four strokes of a cycle, count to yourself, one hundred and one, one hundred and two, one hundred and three, one hundred and four. Pronounce each syllable in your mind clearly and distinctly. After a few stroppings, the rhythm will establish itself subconsciously.

To strop a knife that has been properly cut and is in register with its back requires about one-tenth of the man-hours of effort that are wasted every year in laboratories by trying to strop a knife by guesswork. Stropping is a light, delicate operation and not a virtuoso exhibition of gymnastic prowess. If one desires an answer to the rapid slapping of knives on a strop that barbershop practice has bequeathed to the laboratory, collect a few barbers' razors and view their edges in the microscope.

The use of rouge on the strop is a matter of some controversy. After about twenty years of careful observation, no definite conclusions are available for inclusion in this book. The surface of a proper Shell horsehide strop is too dense to allow impregnation with any substance, nor is any needed. Where rouge is used on a strop, if the color of rouge shows at all, enough is present. Rouge cuts steel so rapidly that it should be confined to those experienced enough to exercise the restraint necessary in its use. In the hands of most technicians, rouge will produce an uncontrollable wire edge which becomes worse with each effort to remove it.

Most laboratory practices have developed around the means available, at the time, for their accomplishment. In a conservative atmosphere, one tends to adhere to a practice long after the necessity ceases or its continuance can be justified.

When a knife has been honed to an unknown angle and stropped until its edge is well rounded, it is necessary, in order to clear the block, that the blade shall lean at an excessive angle as shown in Fig. 56.

In consequence of this advanced position, the cutting side of a well-rounded edge makes contact with the block at an angle little short of 90 degrees. This results in greater displacement of the medium at the instant of parting and increased compression of the sections as they are forced to change direction abruptly.

Fig. 48.

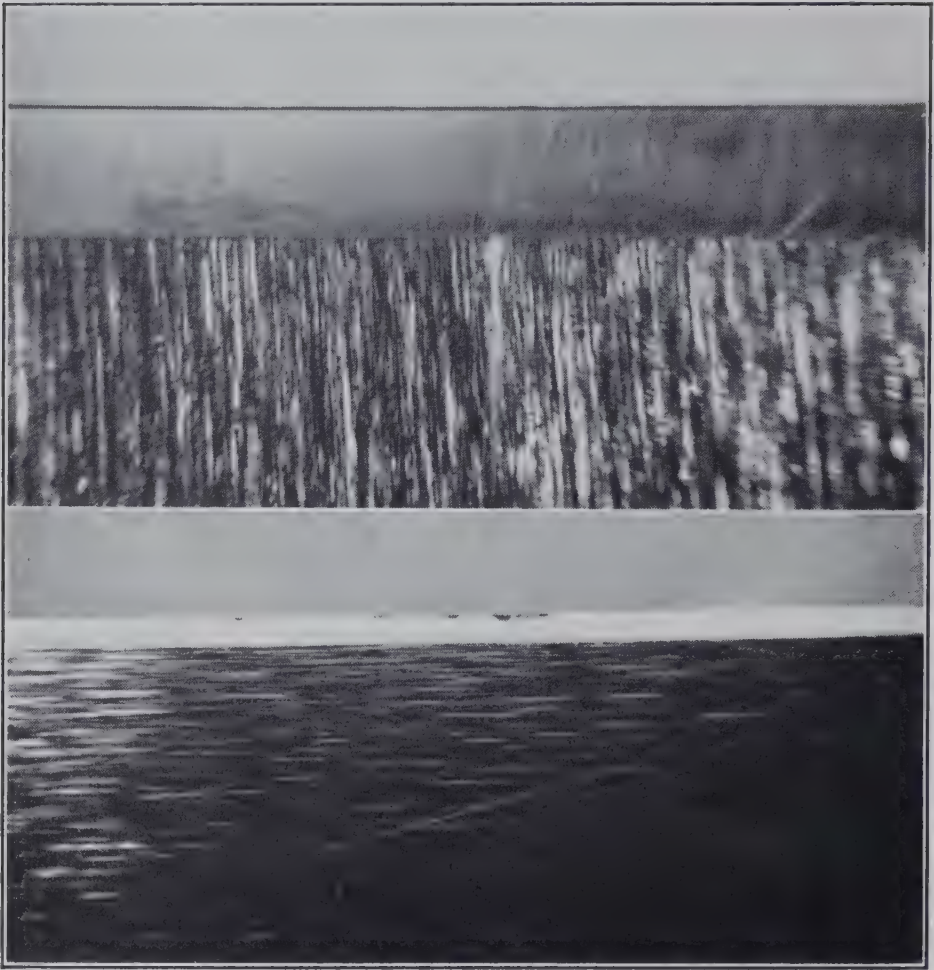


Fig. 49.

A properly sharpened knife will tend to minimize these faults. Many technicians, however, even when provided with a properly sharpened knife, tend to continue the practice of leaning the knife as in Fig. 56, with the results shown in Fig. 49.

Before going farther, let us refer back to the simile of the man pushing up an overhead trap door in a flat roof. Let us suppose that a sudden gust of wind crosses the roof. The man, having

adequate vertical resistance, has nothing but the inertia of his own body to resist a lateral thrust and the trap door is torn from his grasp or forced sideways until his arms reach the confines of the opening. Let us now examine Figs. 50 and 51, a continuous section magnified from Fig. 49.

Fig. 50.

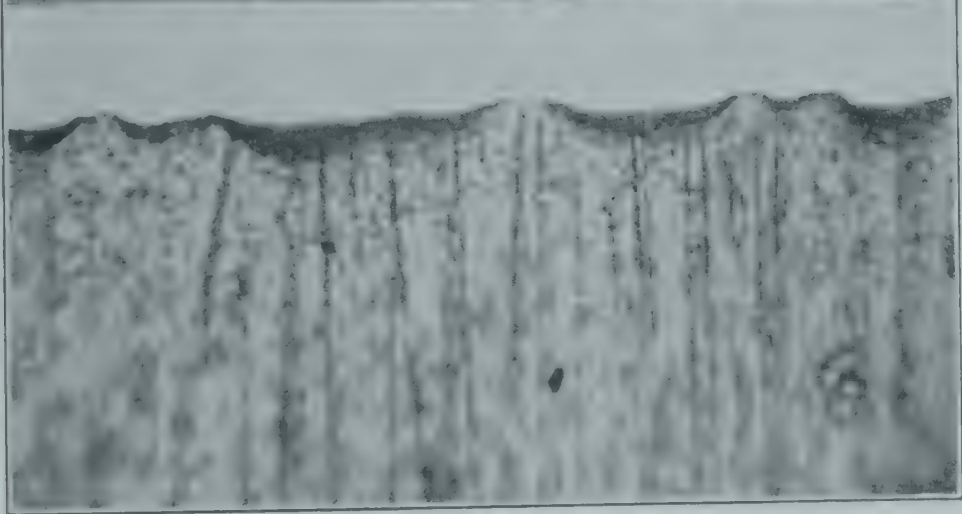
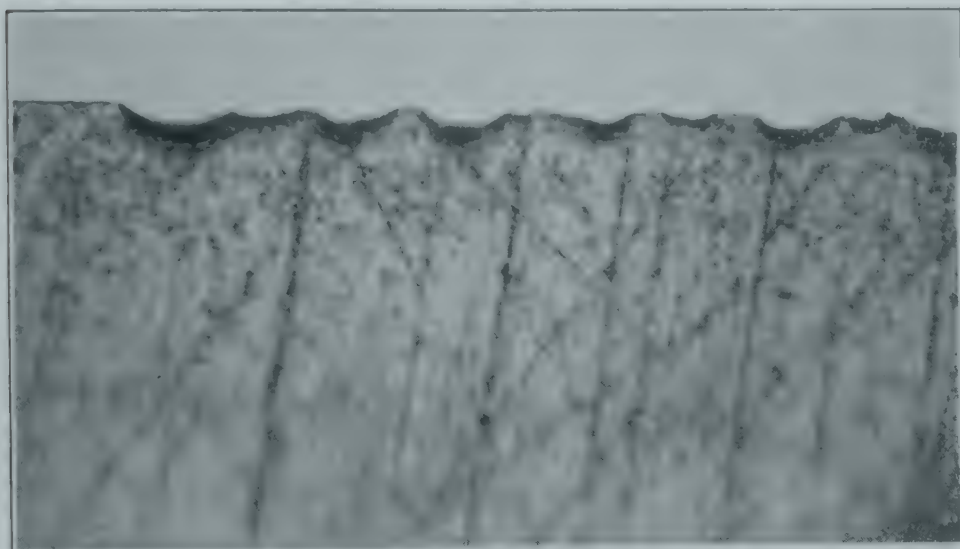


Fig. 51.

The knife, of course, was leaning as in Fig. 56. Upon encountering any hard matter in the specimen, the knife is required to support a secondary stress tangential to its axis. These two stresses do not resolve themselves vectorially. The force expends itself in the direction of travel, chipping a hard knife or stretching and distending a soft knife into a series of pockets resembling small

sugar scoops. As may be seen, the lip of each scoop extends beyond the cutting edge to a greater distance than the thickness of several sections.

Fig. 52.

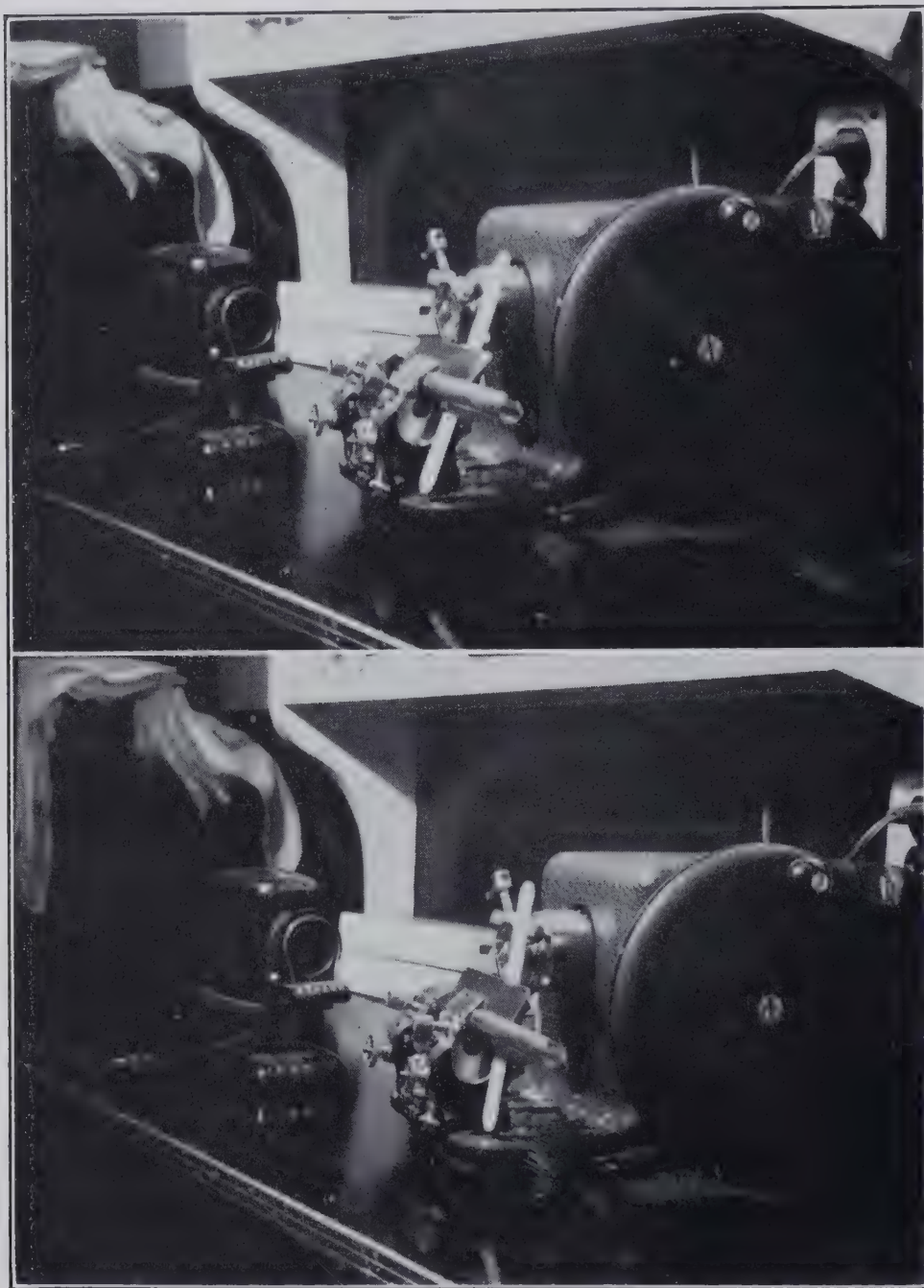


Fig. 53.

In determining the correct cutting position of a knife that has been honed to an unknown angle and stopped by guesswork, one person's guess is as good as another's. Where the back determines

the angle, the position may be established with reasonable accuracy. Fig. 52 shows the same knife as in Fig. 56 and at the same angle as was found to be in use at the time.

Fig. 54.

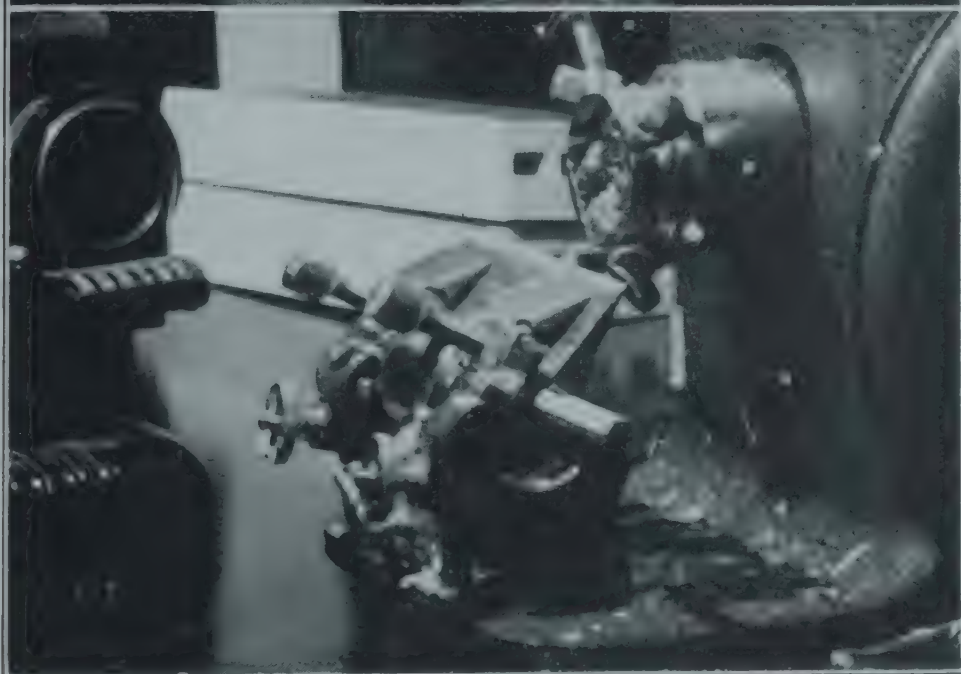
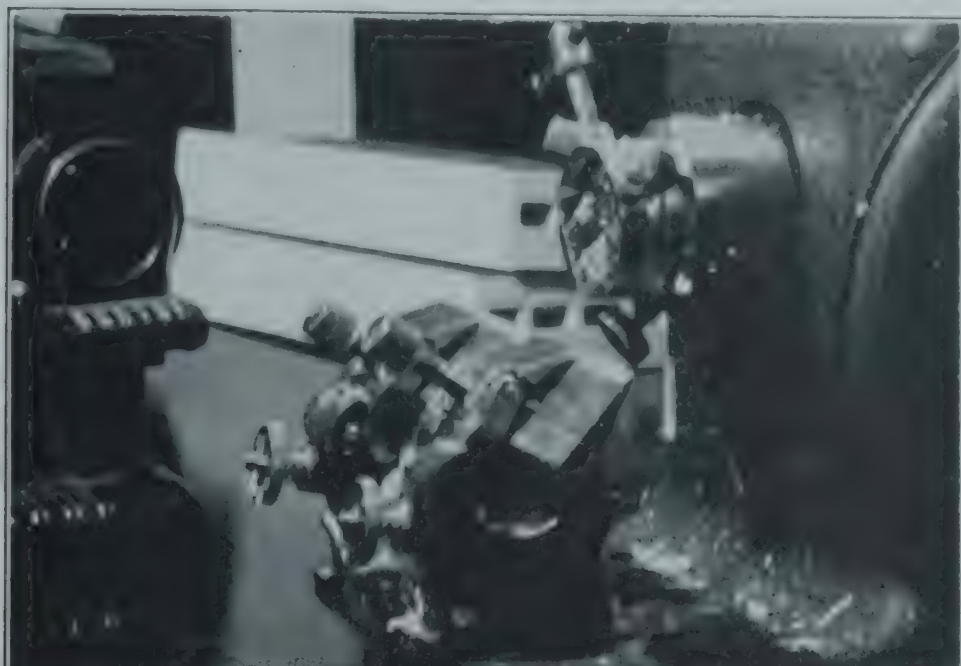


Fig. 55.

By moving the knife into the right-hand clamp and slipping the back on part way, it enabled a tongue depressor to be held from back to bevel. This will establish the exact relationship of the

bevel to the block. With the tongue depressor exactly vertical, the bevel will be exactly flat against the block with no clearance for the cutting edge. Note in Fig. 52 the excessive lean of the blade and the side stress that will have to be borne in thus "scraping" the block.

In Fig. 53, note that the clamp on the right side has been rotated in its trunnion until the tongue depressor assumes a "reasonable" angle. "Reasonable" is the only criterion for a correct angle. A blade cut to a true wedge will require very little clearance but, as the extreme edge becomes rounded by successive stoppings, some additional allowance must be made.

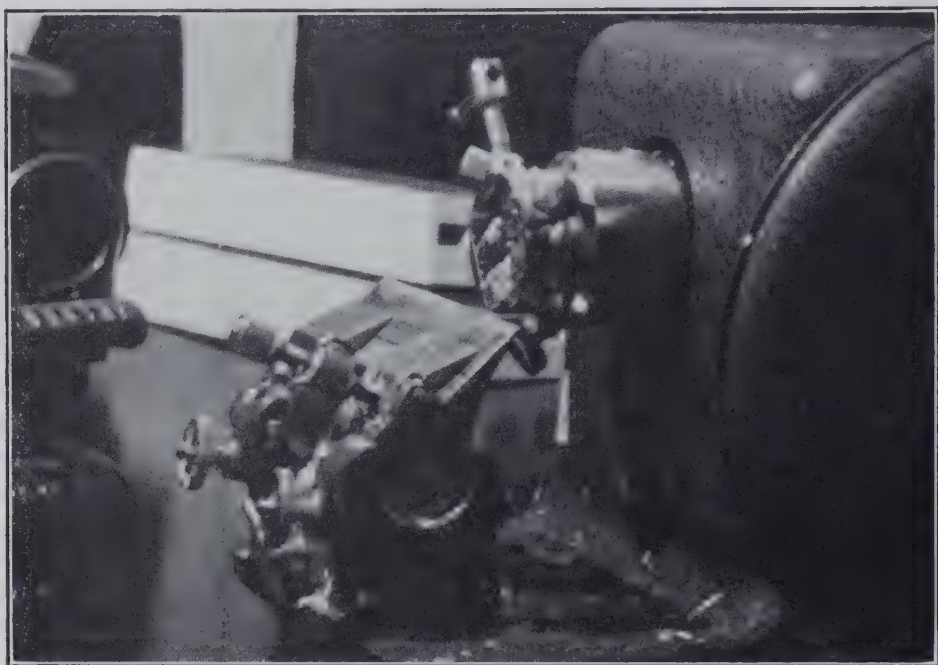


Fig. 56.

It is surprising how many knives have been observed with the edge crushed and broken by the clamps because the knife sat too low in the holder, as in Fig. 54, thus forcing all the work to be done in the center of the knife, the only unspoiled area.

The manufacturer has provided for this condition by means of adjusting screws in the base of the holder. Many technicians, when questioned, said that they did not know they were there; others said that they did not know what they were for. Some said they were adjusted for their widest knife and it was too much trouble to keep changing them. In some, the screws were missing and, in others, rusted solid.

Some technicians have adopted the compromise of adjusting the screws for their widest knives and slipping a round bar (a square bar will not work) under the narrower knives. It has been noted that some are able to work satisfactorily with a pencil under the knife as in Fig. 55.

It has also been observed that many have had resort to the very simple expedient of merely letting the knife rest upon the shank of the clamp tightening screws as is shown in Fig. 56. The advisability of this practice is not within the province of this discussion. It probably would depend upon the actual width of the knife, its relative overhang beyond the clamps determining its stability.

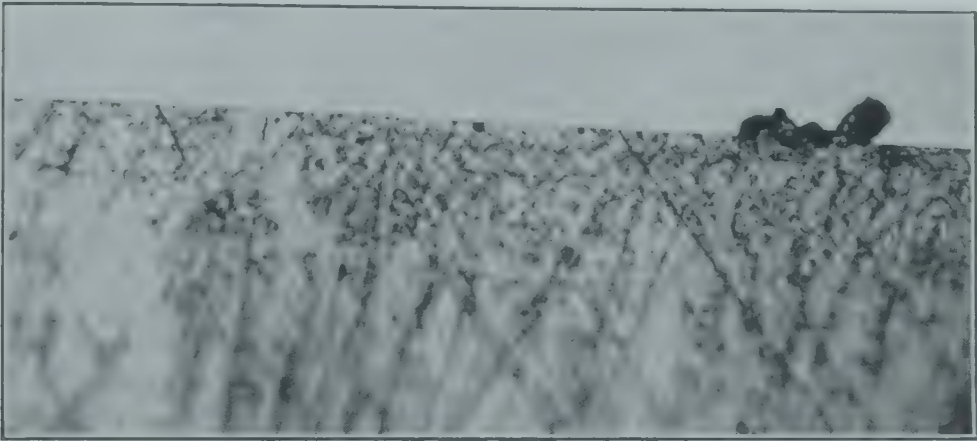


Fig. 57.

While on the subject of clamps, observe Fig. 57. The piece of lint was attracted by static after focusing. This picture is typical of hundreds of knives returned for resharpening to a company that specializes in that work and shows a good cutting edge left unused at both ends of the blade.

The suggestion is made that the manufacturers of microtomes might so alter or redesign the clamps that the entire blade would readily be accessible for use. A single clamp, $4\frac{1}{2}$ inches long, with its trunnions moved forward and thinner sections of stronger alloys might be feasible. If all knives cut more nearly parallel to their axes instead of scraping the block, the stress on the clamps would be reduced proportionally, a nearly vertical thrust presenting simple problems to the designer.

Such a clamp greatly would enhance the useful life of each sharpening and, by reducing sharpenings, enhance the life of the

blade. Any laboratory would be justified in discarding their present clamps and purchasing others that permitted more sections to be cut per sharpening.

In conclusion, do not hold your knife horizontally when returning it to its box. Many recent boxes have a continuous wooden knife slot and if you hit the knife against the slot once in ten times, that is once too often. Place one end of the knife in the slot as in Fig. 58. This will tend to align the knife both vertically and longitudinally and it may then be lowered without damage.*



Fig. 58.

Elimination of Static Formation in Cutting Paraffin Tissue Sections in Dry Weather

The cutting of paraffin tissue sections in dry weather is often difficult or impossible due to static formation because:

1. The sections stick to the knife.
2. When lifting sections from the knife, they are often attracted and stick to metal parts of the microtome.
3. When removing ribbon sections, they tear apart causing loss of many of the serial sections.
4. Sections are more compressed and distorted.
5. Ribbons will not spread flat on the table or slide.

*End of Ratty contribution.

These vexing difficulties are caused by static electricity forming on the surface of the paraffin block due to friction of the knife on the paraffin block during the upstroke.



Fig. 59.—(Courtesy E. Machlett & Son.)

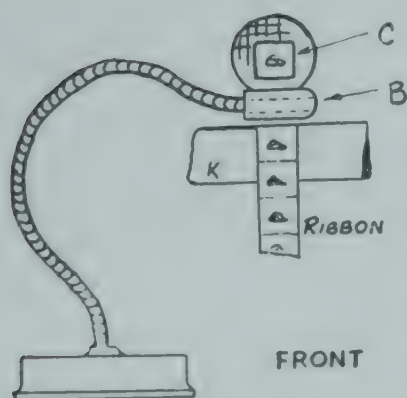


Fig. 60.

Figs. 60 and 61.—(Courtesy E. Machlett & Son.)

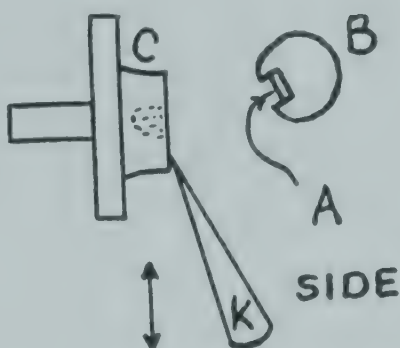


Fig. 61.

In order to dissipate static electricity instantly, the Reco Neutra-Stat* has been put on the market by E. Machlett & Son.

An alpha radiating static eliminator strip (Fig. 61, *A*) is affixed within the slot of a rotatable head (Figs. 60 and 61, *B*). By con-

*No. 61-579 Reco Neutra-Stat, Model M, for microtomes is sold by E. Machlett & Son, 220 East 23rd St., New York 10, N. Y.

torting the flexible tube supporting the head, the static eliminator may be directed toward the paraffin block (Figs. 60 and 61, *C*) and knife (Figs. 60 and 61, *K*).

Irradiating the air with alpha particles, the static eliminator ionizes the air, discharging static charges from near-by surfaces.

It is thus possible to cut completely neutral tissue sections which will not stick to the knife. Ribbons are limp and easily handled. Compression or distortion of sections is reduced.

This equipment is placed to one side of the microtome. It is easily moved out of the way to change paraffin blocks or to make adjustments. The life of the static eliminator is eighteen months or more. Easily attachable replacement heads are available.

The Frozen Section Method Preparation of Tissue

1. *Blocks of Tissue*.—In order to have the fixing solution penetrate thoroughly, it is essential that the blocks be fairly regular and thin.

2. *Fixing Solution*.—The blocks should be immersed in a 15 per cent neutral commercial formaldehyde (40 per cent) for 24 hours. This kills bacteria and initiates hardening and preservation of the tissue.

3. *Trimming Blocks*.—After 24 hours' fixation the blocks should be trimmed to about 1.5 to 1.75 cm. square by 3 to 5 mm. thickness, and placed in 10 per cent neutral commercial formaldehyde. (Neutrality of formaldehyde is very important in order to preserve the staining properties of the nuclei. This is accomplished by addition of 5 Gm. calcium carbonate or bicarbonate of soda to a gallon of stock commercial formaldehyde.)

Sectioning of Tissue

1. *Washing Blocks*.—The blocks are placed in a beaker (250 c.c.) of tap water to remove the formaldehyde, which otherwise not only is detrimental to the skin of the fingers, but also tends to prevent the block from adhering to the holder. If left in water too long, i.e., several hours, the tissue softens and good sections cannot be obtained.

2. *Orienting the Block*.—A block is placed by means of forceps on the block holder as follows:

- a. A drop of water is placed on the holder.

b. The edge of the block facing the knife is placed parallel to the cutting edge.

c. The smaller width of the block faces the cutting edge of the knife.

d. If a capsule, skin, or mucous membrane is present, it faces the knife either on the shorter width or on the left on the longer, so that the cut is made from the capsule into the tissue; otherwise the capsule may be torn off.

3. *Freezing the Block.*—Place the index finger on the block and turn on carbon-dioxide gas slightly for the temporary holding. (Too much gas results in waste and too rapid freezing.) Press down firmly all around to fix to the holder, and release more gas gently until the freezing line, which can be noted on the side of the block, has passed upward through about one-half of its depth.*

4. *Preparation for Cutting.*—Cut off the upper one-third of the block, i.e., the unfrozen portion, and save for further usage. Then shave down to establish a smooth, level surface on which to begin sectioning.

a. If the block is small, it should be frozen throughout and sectioning begun by shaving off the top.

b. If the tissue is too hard, it is likewise well to begin shaving from the top of the block lest the latter be dislodged.

5. *Cutting the Section.*—

a. *Temperature of the block:* Although the temperature cannot be measured, it is an important factor, and experience alone will teach one to recognize the best temperature for ideal sectioning. If the surface to be cut is frozen too firmly, i.e., hard and white, the sections will be fragmentary and tend to disintegrate when placed in water. On the other hand, if the surface is too soft, sections will likewise show fragmentation and contain holes; or if very soft, will disintegrate completely in water. The ideal to be reached is a medium hard, pliable surface, so that a section cut therefrom will show no tendency to break as it piles up over the cutting edge of the knife.

b. *Operating the microtome†:* It is very important that the cutting be done by placing the hand on the rim of the flywheel and turning

*Note.—Even freezing is essential to prevent holes in the section, as the unfrozen portion will break. Hence it is important to press the block to the holder firmly, releasing the gas slowly.

†Bausch & Lomb Optical Co. freezing microtome.

it slowly and evenly to cut a smooth, regular section. If the handle of the flywheel is used and the cutting done automatically and rapidly, the sections are cut too fast and tend to curl or break into shreds. Such speed does not allow sufficient time to adjust the tissue surface to the proper temperature and consistency; in other words, the slower procedure permits warming of the cut surface of the block, which alone is the controlling factor in procuring a pliable section, free from undulations. This pliability prevents disintegration of section when placed in water.

c. On the average, 3 notches indicate the section will be about 10 microns in thickness (1 notch, about 3 microns), due to freezing and thawing action. However, variations in tissues cause variations in thickness of cut sections, e.g., if the lung and uterus tissues are each cut at 3 notches, the latter will be close to only 2 notches thick, because the tissue is more firm, offers greater resistance, and tends to raise the edge of the knife. Hence, it may be necessary at times to use 4 or 5 notches.

6. *Uneven Sections*.—These are usually the result of freezing the block too hard, applying too much speed and unnecessary pressure, especially on firmer tissues.

Removing Section From Knife

1. Wet the tip of the little or ring finger with water, and carefully remove the section from the edge of the knife and place in water.

Keep the knife edge dry. Water left on the edge thaws the tissue it contacts, causing a hole in succeeding sections.

Variations in Tissues

1. *Fatty or Muroid Tissues*.—

a. Cut 15 microns thick.

b. Leave section on edge of knife.

c. Apply 2 or 3 drops of 95 per cent alcohol to dissolve the fat (allow about 1 minute).

d. Remove section with tip of finger and place in water where the surface tension will cause the fatty and muroid tissue to spread out.

e. If this procedure is not satisfactory, it is sometimes helpful to place the section in alcohol for 30 to 45 minutes and then into water.

f. If there is still difficulty, place the whole block in equal parts of acetone and 95 per cent alcohol for 24 hours, reflux in 10 per cent formaldehyde, and proceed as above.

2. *Aorta, Intestine, and Stomach.*—

a. Cross sections are the rule.

b. Orientation of blocks.

(1) Aorta with intimal surface to the left.

(2) Tissues with serosal surface to the left.

c. The block has but a small base and freezing surface, hence freeze three-quarters of the block and begin shaving off the very top of block for sections.

Transferring Section Onto Slide

1. *Apparatus and Procedure.*—

a. *Preparation of slide:* Cleanse in 0.5 per cent acid alcohol and wipe dry.

b. A large water basin, about 7 cm. deep and 15 cm. in diameter, containing about 5 or 6 cm. of water, for floating the cut sections is set upon a background, the color of which contrasts with that of the section. This is usually black, since the tissue is usually light in color.

c. *Reflection of light:* The operator should be seated so as to prevent any direct reflection from the slide to the eyes.

d. (Glass rod (pointed) for transferring section onto slide.

(1) Desirable for soft tissue, as it prevents tearing the tissue sections.

(2) Used whenever metals are to be avoided.

e. (1) Needle may be desirable for sections that are very small or firm.

(2) This should be used neither on sections to be stained for iron nor in the silvering methods.

(3) Care must be exercised as soft tissues are easily torn by the needle, especially if the point is hooked.

2. *Anchoring the Section.* Hold the slide at about a 45 degree angle, with three-quarters of its length under water in such a way that the edge of the water basin affords support to either the slide or the fingers, and thereby aids in steadying the slide. With the glass rod pick up a good section by its midportion, bringing it to the

surface of the water immediately above the center of the slide. When the section is centered, hold one corner at the upper and farther side of the slide in place with the glass rod, and slowly raise the slide out of water, always keeping the farther edge of the slide slightly higher. Do not try to straighten out all folds under water. To eliminate any folds which may be present, place the edge of the slide with the folded portion of the section under water and with the aid of the water and the use of the glass rod if necessary the folds are readily eliminated. Care must be exercised in this procedure; first, keep the edge of the slide above the water wiped dry; and second, keep the upper edge of the section above the water so that the section will not float away from the center of the slide.

After the folds have been removed, tilt the slide against a staining dish at a 45 degree angle, face down, so that the water may partially drain. Do not permit it to dry, as drying may distort the section and shrink the cells. Time for draining depends upon the humidity of the atmosphere; this is usually from 15 seconds to a minute or more.

3. *Dehydrating Section.*—Hold the slide at a slight angle, and drop at short range 2 drops of isopropanol (anhydrous) or absolute alcohol on the section to prevent its breaking. Blow breath over the section until alcohol appears evaporated (or the section becomes white), then blot it twice with filter paper, Whatman No. 2 or similar quality. Next flood the section with isopropanol or absolute alcohol for dehydration, 1 to 10 minutes. Pour off the alcohol, being careful not to disturb the section. In fatty tissues, such as liver, brain, etc., fat is dissolved by alcohol and the section may tend to slip away from the slide. If this happens, blow over the surface of the section gently and remove the remaining alcohol by evaporation to keep section on the slide.

Blot twice with filter paper, then dip twice (in and out rapidly) into a jar of thin celloidin, containing 1 per cent saturated alcoholic solution of gum mastic. The addition of the latter gives a smoother film and prevents rapid deterioration of the celloidin. Blow gently over the surface and place immediately in staining solution.

Sections of mucoid tissue, after being properly drained off, are dehydrated with isopropanol for 5 minutes, then without blotting are dipped in celloidin (to avoid sticking to filter paper), dried by blowing over surface, and stained.

Technic for the Preparation of Large Frozen Sections

A sliding microtome made by E. Leitz, Inc., with large freezing table, is used.

Slides are photographic plates 9×12 cm. Cover glasses slightly smaller, also furnished by Leitz, are used. These are approximately $8\frac{1}{2} \times 11$ cm. Staining dishes are rectangular-shaped museum jars. There should be about a dozen of these. Tissues should be formaldehyde fixed.

Follow the procedure described for routine frozen section in detail.

Staining

1. Place section in alum hematoxylin (Harris or Delafield) for 3 to 5 minutes.
2. Rinse and place in tap water until blue (1 to 2 minutes).
3. Destain in acid alcohol (1 c.c. concentrated hydrochloric acid in 100 c.c. 70 per cent alcohol); dip in and out for even destaining, and stop when no more color leaves the section.
4. Place in tap water for a few seconds to a few minutes.
5. Dip once or twice in 2 per cent ammonia water (2 c.c. strong ammonium hydroxide in 100 c.c. tap water) until section is blue.
6. Dip into tap water, wipe the back of the slide and around the section with towel to remove water.
7. Leave section in 95 per cent ethyl alcohol or 88 per cent isopropanol, 1 to 10 minutes. Holding slide level in hand, add 2 or 3 drops of anhydrous isopropanol or absolute alcohol. Repeat this twice for complete dehydration.
8. Dip in eosinol (eosin in carbolxylol) several times, until the background is stained evenly red. This requires 10 to 30 seconds, depending upon the strength of the solution.
9. Place in carbolxylol for 3 or 4 minutes.
10. Transfer to first xylol for 2 minutes.
11. Second xylol for 2 minutes.
12. Third xylol for 2 minutes.
13. Mount in neutral gum dammar.

Note.—(1) When transferring sections from one solution to another, care should be exercised to drain off as much of the solution as possible, especially from carbolxylol. Small traces of carboic acid carried in xylols will cause fading of nuclear stain.

(2) Ammonia water, carbolxylol, and fresh xylol sometimes swell the sections of liver, brain, cartilage, and spleen, and cause them to fall off. Should this occur, flood section with isopropanol for 2 to 5 minutes, pour off, blow over the section until dry, blot, and dip twice in celloidin solution.

(3) In case the absolute alcohol or anhydrous isopropanol dissolves the celloidin and loosens the section, blot, and dip again in celloidin as previously.

(4) Thin celloidin is prepared by dissolving about 7 c.c. of thick celloidin in 93 c.c. of equal parts of absolute ethyl alcohol and ether, and adding 1 c.c. of saturated alcoholic solution of gum mastic.

(5) Gum mastic (saturated). Twenty-five grams of resin are dissolved in 35 c.c. of absolute or 95 per cent alcohol. The solution will clear within a week, the undissolved portion settling to the bottom of container.

(6) Gum dammar is a saturated solution in neutral xylol. Dissolve resin in histological xylol (especially prepared by Eastman Kodak Company) by placing the mixture in an incubator and shaking it several times a day until it is of heavy syrupy consistency. Then filter through four layers of gauze and store in a brown bottle. If the solution is found to be too thin for mounting purposes, place the unstoppered bottle in an incubator until it is of right consistency.

(7) Compared with ethyl alcohol, isopropanol is very economical for dehydration purposes, and is obtainable through supply houses or direct from the Union Carbide and Carbon Company.

Method of Making Rapid Frozen Sections

Biopsies removed during operation are brought to the laboratory to be examined for the presence or absence of malignancy in cases where rapid diagnosis is of prime importance.

Thin pieces of tissue are placed in a 50 c.c. Pyrex tube containing about 20 c.c. of 10 per cent formaldehyde and boiled immediately over a free flame for 15 to 30 seconds. The formalin is then poured off, the tissue washed thoroughly in tap water, and the sections made on the freezing microtome. The section is attached to the slide in the usual manner and stained in either Harris' or Delafield's hematoxylin by dipping it in and out. Wash section quickly in water, decolorize in acid alcohol, dip in ammonia water, dehydrate by pouring on absolute alcohol or anhydrous isopropanol, and

counterstain in eosinol (described elsewhere) for 15 to 20 seconds; blot in fine filter paper. Now clear in xylol for a few seconds and mount in gum dammar.

This method has some distinct advantages over other methods, and only one feature that might be termed a disadvantage. One gets a well-differentiated stain, making the diagnosis much more dependable than from any other method of rapid sectioning. The slide is permanent and can be kept for future reference and check. It takes only a moment longer than other methods, which is seldom a disadvantage.

For autopsy diagnosis such great haste is not required, and generally from 30 to 45 minutes is allowed for the preparation of frozen sections of fresh unfixed tissues.

The fresh autopsy material is trimmed into small thin blocks. A 10 per cent formaldehyde solution is brought to a boil in a Pyrex tube and tissues are dropped into this hot solution. The tube is placed in the paraffin oven at 55° C. for 15 minutes; then the sections are cut, stained, and mounted in the usual manner.

Frozen Sections Prepared by Use of "Dry Ice"*

The value of frozen sections of pathological tissue is being more and more emphasized and is becoming more generally recognized as the application of this form of examination is demonstrated in many of the large clinics and as it is familiarized by frequent and constant employment in one's own laboratory.

Modification of the technic with resulting simplifications of the process and reduction of cost will, we believe, lead to even more extensive usefulness and remove some of the objections that doubtless occur to many laboratory men. A departure from the standard type of apparatus, with the purpose of eliminating some of the difficulties, is offered.

The special equipment, which may be readily attached to any microtome, consists of an "ice" chamber with a freezing plate, and a plunger with which the "ice" may be brought into contact with or released from the plate. The freezing material is the commercial "dry ice," readily obtained from ice cream packers at a small cost. A block of this substance weighing five pounds may be kept in the cardboard container in which it is delivered and at ordinary room

*Janyier W. Lindsay, M.D., E. Clarence Rice, M.D., and M. A. Selinger, M.D.,
Pathological Laboratory, Garfield Memorial Hospital, Washington, D. C.

temperature for forty-eight hours. By the use of thoroughly insulated containers, it is hoped to extend the life of a supply beyond this time. With this time limit, however, it is seen that many possibilities for preparing sections by the freezing method, at long distances from the hospital or even from the city, are offered. No other equipment than the fixing and staining materials, a small block of "dry ice," and a small portable microtome is necessary. The entire apparatus and materials may be carried in a small case.

The ease of transportation, the absence of noise, the small space occupied, the rapidity of freezing and ready control of the hardness of the specimen seem to us to commend the proposed technic to the plan, which is growing in favor, of freezing sections in the operating room so that the results of the examination may be coordinated with the clinical picture and demonstrated at once to the attending physician and surgeon.

The simplicity of the apparatus and low cost of the freezing material will make it readily possible to utilize the frozen section for an unlimited number of specimens, including those from necropsies.

Terry's Rapid Fresh Tissue Method*

1. Fresh tissue, the cells of which must be alive (i.e., if not kept in the ice chest, no more than 2 hours out of the body) and in bits of not more than 2 by 10 by 10 mm., is frozen on a freezing microtome, and sections cut 5 to 15 microns thick.

2. Remove the sections from the knife with the tip of the finger and allow them to thaw thereon, thus avoiding later development of air bubbles.

3. Unroll the sections with camel's-hair brush in 1 per cent salt solution.

4. Stain 10 to 20 seconds in Unna's polychrome methylene blue.

5. Wash out momentarily in 1 per cent salt solution.

6. Mount in Bruns' glucose medium. Formula: Glucose 240 c.c., distilled water 840 c.c., spirits of camphor 60 c.c., glycerin 60 c.c. Filter.

Bausch and Lomb freezing microtome, with carbon-dioxide attachment, gives excellent sections. Handle the sections from the first salt solution through to the slide with a small glass rod lifter. Keep the sections constantly moving while in the stain. The smaller the

*J. Lab. & Clin. Med., March, 1929.

stain cup, the more readily tissue may be found in it if dropped from the lifter. If fluids for unstained sections are in clear glass over a black background, and those for stained sections are in white porcelain or clear glass over a white background, the work will be greatly facilitated. The various tissue elements are sharply contrasted in red, purple, and dark blue. Even mitotic figures, where present, are beautifully shown. The sections, of course, fade as they lie and decompose.

Embedding Methods for Frozen Sections

The embedding methods for frozen sections are intended for fragmented and loose textured tissues, such as curettings, brain abscesses, and body fluids.

The embedding mass is a mixture of gelatin and agar. Ten grams of powdered gelatin and 3 Gm. of Bacto agar are thoroughly mixed and dissolved in 100 c.c. of boiling water to which is added 0.5 c.c. carbolic acid. This keeps well in a stoppered bottle.

The tissue to be embedded should be previously fixed in 10 per cent formaldehyde. To embed the tissue, the mass is liquefied in a boiling water bath. The tissue, after being dried with a towel, is placed and arranged in a paper box containing the liquefied mass and incubated at 50° C. for 2 hours to allow penetration. The pool is then allowed to solidify in a cool place or in a refrigerator for 2 hours or longer, after which the blocks are removed and trimmed. Frozen sections are made from this block after further fixation in 10 per cent formaldehyde for 3 hours or longer.

These sections are attached, stained, cleared, and mounted in the same manner as ordinary frozen sections.

Serumizing Method

(Krajian)

The method of serumizing is intended for fragmented and loose-textured tissues.

Such tissues are first fixed in 10 per cent formaldehyde. Thin blocks are trimmed and placed in a small Stender dish containing uncontaminated human or animal serum (fresh Wassermann sera). The amount of the serum should be ample to cover all tissues, and the dish (with cover on) should be placed in a warm place overnight (the top of paraffin oven is a good place for it).



Fig. 62.—1, Sections from a case of carcinoma of liver by regular frozen section method. Note the fragmentation of tissue. 2, Sections of the same blocks serumized as described in the method.

Decant the serum and add sufficient dioxane to cover the blocks, and let soak for 3 to 5 hours to cause complete coagulation. Then fix in 10 per cent cold formaldehyde overnight or in hot formaldehyde in paraffin oven at 56° C. for 2 hours. They are then ready for frozen sections.

Paraffin Embedding and Sectioning Methods

Paraffin embedding is useful when very thin sections are desired. To obtain good results, tissue blocks should be small, preferably not over 2 to 3 mm. thick. Before they can be embedded in paraffin it is necessary to dehydrate them thoroughly in graded alcohols, beginning with 70 per cent, and finally with absolute alcohol. The time varies considerably according to the size and consistency of the tissue. The amount of alcohol should be much greater than the bulk of the object.

The alcohols of various strength should be kept in pint or quart bottles. They are prepared by diluting 95 per cent alcohol, and not absolute alcohol, the latter being far more costly. Considerable time will be saved in the preparation of different grades if each bottle is marked with diamond pencil or ink, so as to indicate the levels to which alcohol and water should be added to make up the required concentration.

Dehydration.—The tissues are placed in the lowest grade alcohol (70 per cent) unless a fixative high in alcohol content has been used, in which case dehydration is begun with 90 per cent alcohol. The reason for beginning dehydration with lower grades of alcohol is to prevent serious shrinkage of the tissue due to too rapid extraction of water from the cells.

Some workers dehydrate tissues directly with absolute alcohol, using three successive baths, each of only ten times the volume of tissue. They claim that no more shrinkage is caused by this than by the standard method, beginning with 70 per cent alcohol. While this might be true for certain tissues, it certainly is not applicable to all tissues.

Preservation of Tissue.—Tissues should not be kept in the fixative longer than the specified time. If it is desired to keep them for some time before embedding in paraffin, preserve them in 70 per cent alcohol. Lower dilutions of alcohol macerate tissues, while higher grades harden them excessively. Prolonged preservation of specimens in alcohol affects the staining property of the nuclei. Formol-fixed material should be kept in a 10 per cent neutral solution of formaldehyde (40 per cent commercial).

Identification Tags.—The bottle containing the specimen should be labeled with a tag around the neck; it is still safer to place a small pencil-marked label inside the vessel containing the tissue. The use of such a method prevents confusion and mistakes when many different pieces of specimens are handled at the same time. The tag around the neck of the bottle is used not only for the identification number but also for the record of the solutions through which the objects are being passed.

Clearing Process.—After complete dehydration of the tissue and before proceeding with embedding, it is necessary to replace alcohol by some substance in which paraffin is miscible. The reagents commonly employed are chloroform, benzine, xylol, cedar oil, and recently, dioxane and butyl alcohol. The absolute alcohol is discarded and a large volume of clearing reagent is added to the bottle containing the specimen. For a large object the clearing solution should be changed at least once. The time required for clearing the object varies according to the size and consistency of the tissue and the particular clearing reagent used. Xylol rapidly removes alcohol and clears the tissue. Tissues should be kept in xylol for the minimum time only, since prolonged treatment with this reagent causes the object to become brittle.

The advantages of the use of chloroform, as compared to xylol and similar reagents, are that it enables the paraffin in the mixture to partially infiltrate the tissue, and then it rapidly evaporates from the pure paraffin bath. Its hardening effect is very much less than xylol.

Benzine is preferred by some workers to xylol or chloroform because it clears quickly, makes tissue more transparent, and evaporates rapidly from paraffin bath without much hardening.

Cedar oil does not harden the tissue even by prolonged immersion. It clears from 95 per cent alcohol, but it has the disadvantage of slow penetration and subsequent difficulty of elimination in the paraffin bath.

Paraffin Embedding Process.—An electrically controlled paraffin oven (Fig. 20) is needed, temperature of which should be slightly above the melting point of paraffin. A temperature of 56° to 58° C. is sufficient for most purposes.

Paraffins having a high melting point are hard when allowed to cool; those of low melting point are soft. The choice of paraffin of the

right degree of hardness depends on two factors: (1) For the hard objects it is essential to use hard paraffin in order to support the tissues during section-cutting. For soft objects a lower melting point paraffin is employed. (2) The degree of hardness of paraffin block containing the object is dependent on the surrounding temperature. In summer or in tropical climate it is necessary to use a paraffin of a high melting point in order to have the right consistency for sectioning.

For ordinary work in temperate climates a paraffin with a melting point of 48° to 50° C. is satisfactory. For hard objects or for section-cutting in warm weather a paraffin of 55° C. is necessary.

Paraffin of various melting points is furnished by the laboratory supply houses. Parowax is a cheaper product and is satisfactory for soft tissues in temperate climates. A better product has come on the market, called "Tissuemat," which infiltrates and supports the tissue better than ordinary grades of paraffin.

Infiltration of Paraffin.—After tissues are thoroughly cleared with one of the clearing reagents described elsewhere, it is necessary to remove this reagent and infiltrate the tissue with paraffin. This is done by adding an equal amount of melted paraffin to the clearing reagent and placing it in the oven. As the clearing medium warms, it dissolves the paraffin and permeates the objects with the mixture. When the infiltration process is completed, the clearing and paraffin mixture are discarded and the tissues are soaked in melted paraffin (in paraffin oven) which is changed at least once.

The duration of the paraffin bath depends on the size and thickness of the object. It takes a longer time for paraffin to penetrate through large and dense objects than through those which are thin and loose textured.

For successful embedding it is necessary to keep the object in oven just long enough for complete infiltration of paraffin. Any traces of clearing reagents remaining in the tissues, or the failure of the paraffin to penetrate them, indicate insufficient duration of the paraffin bath, and prevent proper section-cutting. On the other hand, prolonged treatment in the paraffin oven causes shrinkage and hardening of tissue, thus making section-cutting very difficult; therefore, complete removal of the clearing reagent from the object requires a sufficient number of changes of paraffin.

Process of Casting.—The tissues are first placed in molds of right size filled with paraffin; next they are placed in the mold with that

side down from which sections are to be cut; and finally, the paraffin containing the tissues is hardened by cooling.

There are several types of boxes for molding paraffin, such as the metal boxes made with pairs of variously sized L-shaped pieces, and hand-folded paper boxes. The L-shaped metal pieces are set one against the other on a glass plate. The shape of the block can be varied with the size of the object.

Procedure.—

1. Fill the boxes with melted paraffin.
2. Place the tissue in the bottom of the paraffin with warmed forceps.
3. Label the boxes with case number.
4. Gently immerse the box in a basin of ice water to cool the paraffin rapidly, or set it on a wet towel.

No attempt should be made to trim the block before paraffin is completely set. Small blocks of tissue should be left in water 15 minutes or longer to insure complete solidification. Large blocks require one-half hour or longer.

General Facts About Paraffin

Special paraffin is manufactured for embedding purposes. Different samples of paraffin have different melting points and also different “plastic points.” Paraffin is a mixture of hydrocarbons which solidifies into characteristic types of crystals, varying to some degree with the proportion of harder and softer hydrocarbons present. The peripheral crystals are oriented with respect to the cooling surface while the center of the mass forms a meshwork. The plastic point is the lowest temperature at which permanent deformation may be made without fracture. A paraffin with low plastic point appears more translucent, is less brittle, but compresses more in sectioning. The hardness of paraffin depends on its plastic point which lies a few, but variable, number of degrees below melting point. Consequently, the plastic or melting point of paraffin has to be adapted to the room temperature where sectioning is being done. In a warm room, higher melting point paraffin must be used than in a colder room. The alternative is to condition the paraffin by the addition of various materials. Bayberry wax, rubber, and other waxes may be added to paraffin to improve its plasticity and cutting qualities.

Warm paraffin shrinks as it cools and compresses the tissue in the block. The tissue that is harder than paraffin withstands this pressure, but soft and spongy tissue may be under considerable pressure. When sectioned, the tissue tends to expand to the shape and size it had before compression, and if confined by the paraffin around it, pleating or wrinkling results. Unless the hardness of the paraffin is adapted to the temperature at which the cutting is done and to the nature of the tissue, good sections may not be expected, regardless of the excellence of the microtome and the knife. When the material has been properly dehydrated, it is possible to re-embed it in another paraffin should the first paraffin prove unsatisfactory. Poorly prepared tissue can rarely be salvaged.

The paraffin embedding methods are as follows:

Chloroform Method

1. Dehydrate tissues in 70 per cent alcohol for 2 to 3 hours.
2. Dehydrate tissues in 80 per cent alcohol for 2 to 3 hours.
3. Dehydrate tissues in 95 per cent alcohol for 2 to 3 hours.
4. Dehydrate tissues in 95 per cent alcohol for 2 to 3 hours.
5. Dehydrate tissues in absolute alcohol for 2 to 3 hours.
6. Chloroform for 2 hours.
7. Chloroform and melted paraffin, equal parts (in paraffin oven at $55^{\circ}\text{C}.$), for 2 to 3 hours.
8. Pure paraffin in paraffin oven at $55^{\circ}\text{C}.$ for 2 to 3 hours.

Benzine Method

1. Dehydrate tissues in 70 per cent alcohol for 2 to 3 hours.
2. Dehydrate tissues in 80 per cent alcohol for 2 to 3 hours.
3. Dehydrate tissues in 95 per cent alcohol for 2 to 3 hours.
4. Dehydrate tissues in 95 per cent alcohol for 2 to 3 hours.
5. Dehydrate tissues in absolute alcohol for 2 to 3 hours.
6. Benzine for 2 to 3 hours.
7. Benzine and melted paraffin, equal parts, in the paraffin oven at $55^{\circ}\text{C}.$ for 2 to 3 hours. For the evaporation of benzine, leave the corks out of the bottles, otherwise the fumes will cause an explosion.
8. Pure melted paraffin in the paraffin oven at $55^{\circ}\text{C}.$ for 2 to 3 hours.
9. Second paraffin bath in paraffin oven at $55^{\circ}\text{C}.$ for 2 to 3 hours.

Benzine and Acetone Method

1. Fix thin blocks of tissue in 10 per cent formaldehyde for 16 to 24 hours. In emergency cases, fix for one-half hour in paraffin oven at 56° C.
2. Acetone for 1 hour.
3. Second acetone for 1 hour.
4. Benzine for 30 minutes.
5. Second benzine for 30 minutes.
6. First paraffin bath for 1 hour.
7. Second paraffin bath for 1 hour.

The above method could advantageously be used for routine surgical material. The quantity of acetone used should be about 20 times the volume of tissue.

Dioxane Method

Fix tissues in any desired fixative in usual manner. Zenker's fixed tissues are washed 16 hours before being subjected to dehydration.

1. Dehydrate in 25 per cent dioxane for 1 hour.
2. Dehydrate in 50 per cent dioxane for 2 hours.
3. Dehydrate in 100 per cent dioxane for 3 hours.
4. Paraffin at 56° C. for 30 minutes.
5. Paraffin at 56° C. for 1 hour.
6. Paraffin at 56° C. for 3 hours.
7. Embed in paper boxes.

Dioxane is highly inflammable, and the vapor is very toxic. Excessive exposure to the vapor will cause serious consequences.

Butyl Alcohol in Paraffin Embedding

Fix tissues in 10 per cent formaldehyde 24 hours or longer.

1. Dehydrate in 80 per cent ethyl alcohol for 3 hours.
2. Dehydrate in 90 per cent ethyl alcohol for 20 hours.
3. Butyl alcohol No. 1 for 3 hours.
4. Butyl alcohol No. 2 for 3 hours.
5. Butyl alcohol No. 3 for 15 hours.
6. Pass to paraffin No. 1 in paraffin oven at 56° C. for 1 hour.
7. Pass to paraffin No. 2 in paraffin oven at 56° C. for 1 hour.
8. Pass to paraffin No. 3 in paraffin oven at 56° C. for 1 hour.
9. Embed in paper boxes.

Embedding With a Water-Soluble Wax

Blank and McCarthy¹ have developed a method for embedding tissues which is suitable for skin, liver, kidney, spleen, lung, testis, muscle, spinal cord, pancreas, adrenal, gastrointestinal tract, etc. It is especially applicable to delicate specimens such as skin with vesicles, eyes, embryonic membranes, etc. This method eliminates time-consuming, deleterious, dehydrating chemicals.

Materials.—

Embedding mixture:

Carbowax 4000	-----	9 parts
Carbowax 1500	-----	1 part

Melt these substances together at 55° C.

Paraffin oven or a thermostatically controlled paraffin bath of the Autotechnicon type.

Solution for floating and affixing sections to slides (optional):

Potassium dichromate	-----	0.2 Gm.
Gelatin	-----	0.2 Gm.
Distilled water	-----	1,000 c.c.

This mixture is boiled in daylight for 5 minutes, cooled, and filtered, and is ready for use.

Routine stains, dehydrating agents, and balsam.

Method.—Tissues fixed and washed in the routine manner are cut into desired sizes. As with other methods, the thinner the specimen the more rapid the impregnation with wax. The tissue is immersed in the molten embedding medium and agitated occasionally. The minimum time for impregnation is 30 minutes for a 1 mm. section, 60 minutes for a 2 mm. section, and proportionately longer times for thicker sections. Specimens have been left in molten Carbowax for periods up to four days with little perceptible distortion. The routine time should be about 3 hours. After impregnation, the specimen is transferred to a mold and fresh molten wax is added to form a block. The mold is transferred to a cool dry place, such as a refrigerator, and allowed to harden for 5 to 10 minutes. Contact with ice and water must be avoided because of the solubility of the wax. The hardened block is attached to a microtome specimen holder and is ready for sectioning. The block may be cut at 2 to 10

¹Blank, Harvey, and McCarthy, Philip L.: J. Lab. & Clin. Med. 36: 776-782, 1950.

microns with the formation of satisfactory ribbons. The knife and block should not be moistened or chilled because best results are obtained at room temperature.

Sections may be hydrated and affixed to the slide by two methods. Use of gelatin-dichromate solution instead of water to float the sections keeps the sections firmly in place on the slide during subsequent staining procedures. This is a timesaving, simple and rapid method for embedding tissues in a water-soluble wax for histological study.

The Technic of Cutting and Mounting Paraffin Sections

The tissue blocks embedded in the paraffin pool (in the paper boxes) are first cut out by trimming away the paper with a scalpel or other sharp knife, and then shaping them into square blocks. At least 1 mm. of paraffin should be allowed to remain around the blocks, and the upper and lower surfaces should be parallel. Next they are placed in pillboxes bearing their proper case numbers, and are stored away in a cool place or refrigerator.

When ready to cut sections, place the blocks in ice water. Fasten one of the blocks to the block holder by heating the latter in the flame of a Bunsen burner for a few seconds, until it just melts the paraffin. Then hold the block of tissue in proper position against it and quickly dip it into the ice water to cool and harden. Carefully adjust the block holder to the microtome. Screw back the feed mechanism. Fix the knife in its proper position and adjust the indicator, regulating the thickness of the sections (the routine histological sections are cut 7 to 10 microns thick; for research purposes, very thin sections, varying from 3 to 5 microns, are required). Next see that both upper and lower surfaces of the paraffin block are parallel to the edge of the knife.

When the above adjustments are made, see that the knife is sharp and in its proper position, and that the paraffin block is of the right consistency. Then by rapid motion of the flywheel cut sections; these will adhere to one another to form a ribbon. The faster the sections are cut, the better they will adhere together. Then hold the free end of the ribbon gently with a fine pair of forceps and pick up and support with a camel's-hair brush the other end of the ribbon. Lay the sections on the surface of a dish of warm water (about 42° C.) and stretch out thoroughly by pulling their ends with the same instruments. Small foldovers and wrinkles can be flattened out by stretching them with a teasing needle.

Have ready some clean slides and a diamond pencil for marking them. Cut the ribbon to the desired lengths. Rub the surface of slide with a drop of egg albumen fixative (see page 99); pick up the first strip of the ribbon on the slide and let the water drain off.



Fig. 63.—The completed "boat," ready for use.

(Figs. 63 to 67 from Gradwohl: Clinical Laboratory Methods and Diagnosis, The C. V. Mosby Company.)



Fig. 64.—Heating the paraffin microtome pivot.

Repeat this process for every strip. Number each slide with its proper case number and place it (in slanting position) in wooden box holding 25 slides. Sometimes it becomes necessary to flatten out sections before they are subjected to the drying process. This



Fig. 65.—Cooling the paraffin block after mounting it on the pivot.

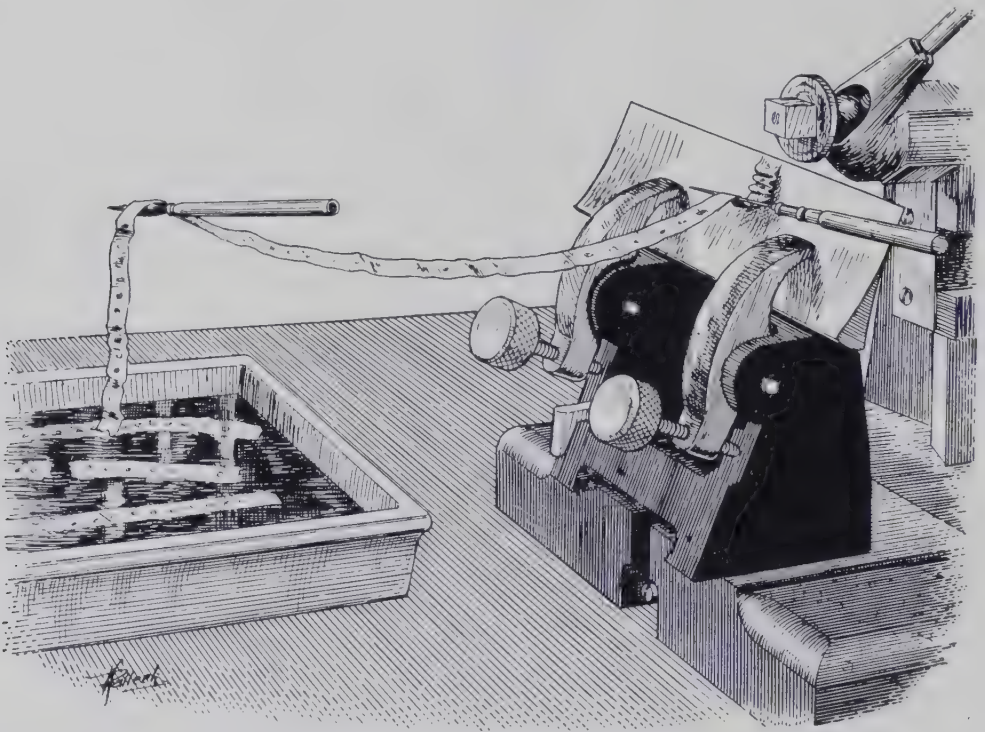


Fig. 66.—Cutting paraffin sections and floating the ribbons on warm water.

is done by holding the sections over a Bunsen burner at a temperature several degrees below the melting point of paraffin (about 45° to 50° C.). Dry the sections by placing them in the paraffin oven at 55° C. for 3 to 5 hours to coagulate the egg albumen fixative, thereby causing them to adhere firmly to the slide. If the sections are to be stained in aqueous solutions for many hours, the drying must be prolonged, otherwise they will fall off. To prevent this, sections fixed with the albumen fixative are dipped in thin celloidin (such as is used to fasten frozen sections to the slide).

When the sections are properly dried, they can be stained by one of the standard staining methods.

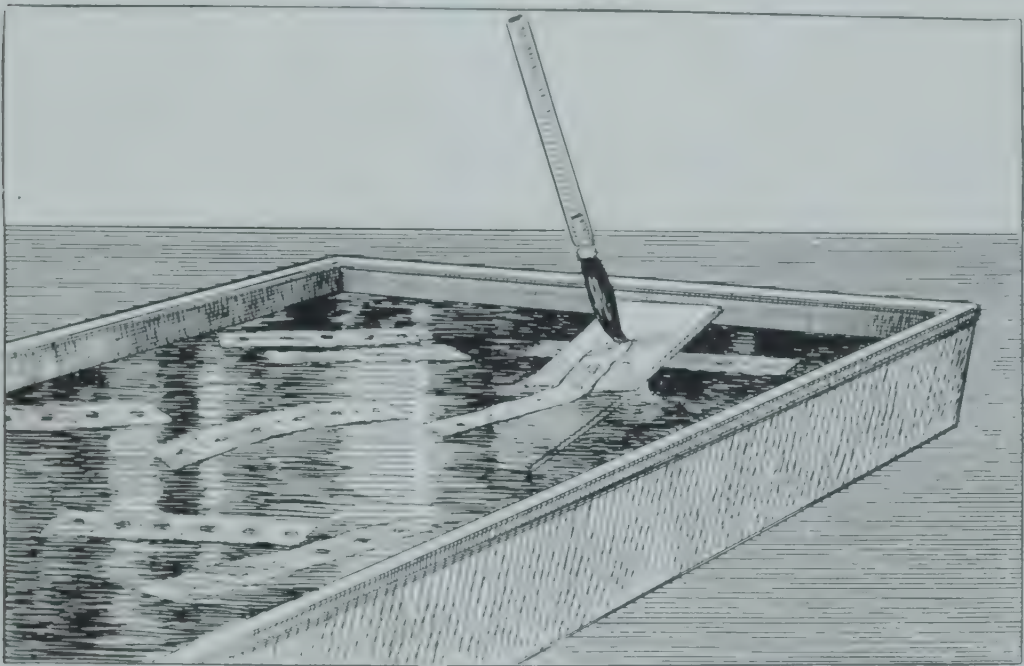


Fig. 67.—Placing paraffin sections on a glass slide.

Difficulties Encountered in Cutting Ribbons

1. Ribbons fail to form. (a) Room is too cold or paraffin is too hard. Use soft (lower melting point) paraffin; warm knife slightly by blowing breath on it, or immersing in warm water; or place a desk lamp so that the light and heat fall on the knife and block. (b) Tilt the knife less. (c) Cut thinner sections. (d) Knife may be too dull—resharpen. (e) Dip block into a softer paraffin and trim so that the thin layer remains on the upper and lower edges of the block. (f) Unroll the section and hold it lightly against the knife with a camel's-hair brush. If the first few sections can be held down, the ribbon will hold and follow.

2. Crooked ribbons. (a) When sections are wedge shaped the sides of the block are not trimmed parallel. (b) Edge of block may not be parallel to knife edge. (c) Try another part of the knife—sometimes irregularities of the knife edge cause crooked ribbons. (d) Or, the paraffin on one side of the block may be softer than at the other side, especially if the material has been re-embedded in a paraffin of different hardness—re-embed the material and stir the melted paraffin.

3. Sections compressed, wrinkled, and jammed together. (a) The knife may be too dull. (b) Or, the room may be too warm; cool trimmed block and knife in very cold or ice water immediately before sectioning, or re-embed in harder paraffin. (c) Tilt the knife more; the knife tilt may be too slight so facet bevel rubs over block. (d) The knife edge may be gummed with paraffin; wipe with cotton moistened with xylol. (e) Soak block in water for 1 or 2 hours before cutting. (f) If cutting is done too rapidly, thin sections will wrinkle.

4. Sections crumble and specimen may tear out. (a) Material incompletely dehydrated or improperly cleared. (b) When soft and mushy, material incompletely infiltrated—re-infiltrate and embed. (c) Alcohol not completely removed by clearing reagent. (d) Object too long in paraffin bath or paraffin too hot. (e) Subject hard and brittle due to clearing reagent. Try toluene in place of xylol or a mixture of toluene and cedar oil. (f) Try celloidin embedding or a rubber mixture with paraffin for fragile material. (g) Try dioxane method for dehydrating.

5. Split ribbon or lengthwise scratches in ribbon. (a) If there are nicks in knife, sharpen or use another sharp knife. (b) Use less tilt of knife so it will cut rather than scrape. (c) Knife edge may be dirty. (d) Object may be too large—use celloidin. (e) Hard particles in block may cause scratching. If there is dirt in the paraffin, filter or decant paraffin. There may be crystals in fixing solutions (mercuric chloride); washing was insufficient. If calcareous particles are present, decalcify.

6. Knife rings on upstroke and sections are scratched. (a) Change knife tilt to greater or lesser degree—tilt must be sufficient to clear facet bevel, but not enough to scrape instead of cut. (b) Material is too hard. Soak in water to soften; clearing may be at

fault. (c) A thicker or wedge-shaped knife may prevent springing of the edge when cutting. (d) Material may be too tough—try celloidin.

7. Sections lifted from knife on upstroke. (a) Increase knife tilt. (b) Room too warm or paraffin too soft—try harder paraffin or cooler room; or cool the block. (c) The knife may be too dull—resharpen.

8. Sections stick to knife. (a) Knife edge dirty. (b) Increase knife tilt. (c) Try a sharper knife.

9. Undulations in the surface of section. (a) Tighten all screws on knife and block holder and see that knife holder is clamped tight to microtome. (b) Lessen excessive knife tilt to prevent vibration.

10. Sections fly and stick to parts of microtome, due to static electricity formed from the friction of cutting. This usually occurs in winter when the air is very dry. (a) Increase the humidity of the room by boiling water in an open pan. (b) Ground microtome to a water pipe with a wire or a chain. (c) Ionize the air by an electrical method.

11. Sections vary in thickness or are skipped. (a) Knife not tilted enough to clear facet or bevel, or tilted too much, and tissue is compressed until the inevitable expansion gives a thick section. (b) Some of the clamping set screws on the block or knife holder are not tight or knife holder block is not clamped firmly. (c) Microtome worn out through lack of lubrication, or not in adjustment.

Mayer's Egg Albumen-Glycerin Mixture

Mayer's egg albumen-glycerin mixture for attaching paraffin sections to the slides is made as follows:

Separate the white of an egg, discarding the yolk. Pour it into a graduate and add an equal amount of glycerin. Then pour the mixture in a beaker and beat it thoroughly with a glass rod. Filter through several layers of clean gauze. To the filtrate add a crystal of thymol or several drops of phenol to prevent decomposition. The mixture will keep for many months in a well-stoppered bottle.

Serial Section Method

To obtain serial sections by either of the three methods, it is only necessary to avoid losing any of the sections cut. The paraffin

method is preferable to the others, because by this method long ribbons are cut and placed on a sheet of paper in proper order. They can then be divided by means of a teasing needle into short series of any desired number of sections and fastened to numbered slides by means of egg albumen-glycerin fixative.

Vacuum Embedding

Vacuum embedding, or embedding under reduced pressure, is used for certain purposes. A special type of thermostat is necessary, in which the embedding chamber is so arranged as to be airtight. The procedure consists in partially reducing the pressure inside the thermostat during the process of impregnation with the paraffin. The necessary reduction in the pressure is effected either by a hand exhaust pump or by a water aspirator.

The advantages of the method are as follows:

1. The time required for embedding is considerably reduced, the clearing reagent being more rapidly replaced by the paraffin. This reduction in time is a great advantage in those tissues which become unduly hard during the process of embedding.
2. Any air present in tissue is extracted.

Apparatus Required.—For the vacuum thermostat the embedding chamber is a circular brass well, closed at its upper end by a thick glass lying upon a rubber ring. The chamber is kept at the proper temperature by a water jacket, while a small tube connects it with the exhausting apparatus (vacuum pump or aspirator). There is also an arrangement in the shape of a small screw plug, fitted into the wall of the chamber for the admission of air. When the apparatus is used in conjunction with water aspirator, a glass bottle or a thick-walled flask of at least 500 c.c. capacity should be placed between aspirator and thermostat. This serves to avoid water being sucked directly into the embedding chamber, should the water supply to the aspirator be inadvertently turned off before opening the air valve. Finally, it is advantageous to attach to the apparatus a mercury manometer.

Procedure.—

1. Place the cleared tissue in pure paraffin within the thermostat.
2. Reduce the pressure very slowly. A reduction to 400 or 500 mm. Hg is usually sufficient. The time required for impregnation

varies from one-quarter to one-half of that required for the same objects at atmospheric pressure.

3. Slowly admit air through the air valve until the pressure within the thermostat is in equilibrium with that of the atmosphere. For large objects, repeat the process of exhaustion two or three times, changing the paraffin each time. To insure success the exhaustion and the readmission of the air must be very gradual. Delicate air-containing tissues are ruined by a too rapid change.

The vacuum method is indicated in the following instances:

1. For air-containing tissues, especially lung tissue.
2. For large and dense objects, e.g., whole embryos or large pieces of brain.
3. For tissues which become unduly hard if the embedding bath is prolonged, e.g., fibrous tissue and muscle.
4. For any work where speed is important.

Technic for Small Pieces of Tissue, Skin, etc., Using Bouin's Solution as Fixative

Bouin's Solution

Saturated aqueous picric acid -----	300 c.c.
Formalin (40 per cent) -----	100 c.c.
Glacial acetic acid -----	20 c.c.

Fix tissue overnight in Bouin's solution (24 hours is the maximum).

First Day

- 9:00 A.M. Wash in 50 per cent alcohol.
- 10:00 A.M. Wash in 70 per cent alcohol containing about 3 drops of a saturated solution of lithium carbonate.
- 12:00 NOON. Put in 70 per cent alcohol (may be left over the weekend in this solution).
- 3:00 P.M. 80 per cent alcohol.
- 5:00 P.M. 95 per cent alcohol.

Second Day

- 9:00 A.M. Absolute alcohol.
- 10:30 A.M. Amyl acetate.
- 4:00 P.M. Paraffin.
- 5:00 P.M. Pour off paraffin and leave tissue outside of paraffin oven. (Never leave tissue in oven overnight.) (Never re-use any paraffin.)

Third Day

- 9:00 A.M. Put in second paraffin.
- 12:00 NOON Put in third paraffin.
- 2:00 P.M. Embed. Cut and stain with routine hematoxylin and eosin stain.

Amyl acetate can be used for clearing formalin-fixed tissues as well as those fixed in Bouin's.

If tissue is fixed in formalin, first wash in water from 9 A.M. until 10:30 A.M.

Then place in 50 per cent alcohol and then 70 per cent alcohol until 12 noon. The technic for Bouin's fixed tissue is followed from this point.

Staining is the same as that already described, hematoxylin, eosinol, etc.

The Autotechnicon Method for Paraffin Sections*

This is a method especially designed for the fixation, dehydration, and embedding of specimens by use of a special automatic apparatus



Fig. 68.—Autotechnicon.

(Figs. 68 to 81 courtesy The Technicon Company.)

called the Autotechnicon. This is an instrument for the automatic fixation, dehydration, infiltration, decalcification, and staining of tissues. It is adaptable to any method or technic and, once set, assures undeviating adherence to that technic, eliminating human error. It is independent of any intervention or supervision. It

*Information furnished by The Technicon Company and Technicon Chemical Company, 215 E. 149th St., New York 51, N. Y.

operates automatically. It consists of a timing clock, reagent beakers of Pyrex glass, beaker platform, mastershift carriage, beaker covers, displacer rotor, receptacle basket, receptacles, washer, and paraffin bath. Fig. 68 shows the equipment.

With this instrument, it is possible to accomplish all the steps of fixation, dehydration, and staining mechanically, and it performs a large volume of work more economically than could be carried on by hand. It is especially recommended for overnight routine and also for weekend tissue routine. To employ this instrument for staining, one simply plugs in a one-hour staining clock, at the same time replacing the processing fluid beakers with others containing those staining fluids required by the prescribed technique. The slides to be stained are placed in the Autotechnicon staining rack, whereupon the same procedure is followed as in processing. In most cases, the slides may be removed, fully stained, within 35 minutes.

Typical Daily Routine in Hospitals Using the Autotechnicon System

During the day, tissues are collected in the Technicon mobile refrigerator. This unit may be wheeled to any location where it can be plugged into an electrical outlet, outside an operating room, in a central corridor adjacent to the operating suite, or in the morgue, as occasion demands. Immediately upon excision, the tissues are placed in the freezer, avoiding any possibility of drying out, necrosis, or general deterioration. The temperature within the refrigerator is maintained at a point just below freezing so that tissues, while ideally preserved, will still be easy to cut and trim on removal.

When the operating or autopsy schedule is over for the day, the unit can be rolled into the pathology laboratory where it is plugged into an electrical outlet again, to store the tissues until they are ready for processing.

At a convenient time, usually in the late afternoon, the pathologist removes the tissues which have been collected during the day. As he prepares and examines each specimen, he describes its gross aspect, dictating his observations into a recording apparatus. It is convenient to have a permanent microphone set up at the cutting board, with a foot switch to start and stop the recorder. In this way, both hands are free for dissecting. While dictating his gross

description, the pathologist cuts pieces of tissue to proper size for processing. (Such pieces should be cut no thicker than 4 mm., regardless of the other dimensions.) As the pieces are cut, they are placed into a tissue receptacle fitted with proper dividers so that it can hold several specimens for simultaneous processing. During this procedure, it is convenient to store receptacle bottoms on one tray, dividers on another, and tops on a third. The pathologist can then select the tissues, pick the proper dividers, place them in the receptacle, and finally snap on the cover in a continuous operation.



Fig. 69.—The displacer rotor is attached to the receptacle basket and the whole assembly mounted on the Autotechnicon.

If the pieces are small, the receptacle may hold three; if larger, two; while very large ones may require the whole receptacle. A paper identification slip (or slips) is placed in the receptacle along with the tissue. Slips should be no larger than 3×10 mm. so as not to impede penetration of the fluid. Notations made with an ordinary graphite pencil will stand up under all the reagents encountered during the ensuing processing, and will still be quite legible when finally they come out of paraffin. As each receptacle is filled, it is placed in the receptacle basket which is immersed in a beaker of fixative lying beside the cutting board. Receptacles should be

placed vertically in the basket, rather than horizontally, for better penetration and draining during process.

When the receptacle basket has been filled, the displacer rotor is attached to it, and the whole assembly, including the beaker of fixative in which it has been lying, is mounted on the Autotechnicon. Next, the technician checks to see that no paraffin adheres to any of the beaker covers (it might solidify, causing the beaker to tip and spill when the carriage rises).

The machine is now started and, from this point on, takes over. Precise time periods for each of the steps in the desired procedure have already been cut into the timing dial.

Assume that the machine has been started at 5 P.M. In a typical routine, the tissues will remain in fixative for 4½ hours, until 9:30 P.M. Recommended for fixation is Technicon Fixative, a stable solution with all the properties of Zenker fluid, yet none of its disadvantages. It is chemically balanced to preserve the selectivity of the ensuing stain. While it is a potassium dichromate formula, tissues fixed in it do not have to be washed as it produces no appreciable precipitate. Moreover, this fixative does not harden tissues as do conventional Zenker's and similar solutions. Tissues may remain in it for prolonged periods, without harmful effects. Subsequent immersion in a beaker of water, or the tissue washer, for a period of 30 minutes is recommended, in order to wash off the excess fixative to avoid contamination of the reagents that follow.

The next step is from 10 P.M. to 4 A.M. During this period, the tissues go through six changes of dehydrants; 1 hour for each change. An ideal agent for this purpose is Technicon Dehydrant, which is neither hygroscopic nor highly volatile. It does not take up atmospheric moisture and there is practically no loss from evaporation. The solution, while presenting excellent dehydrating qualities, does not cause tissue shrinkage, nor does it harden. Thus, the tissues may be left for prolonged immersion, without damage. The physical properties of the compound are such as to materially lessen the fire hazard.

From 4 A.M. to 6 A.M. tissues go through the next steps—two stages (of 1 hour each) of clearing. Many users have found Technicon clearing agent to be an excellent medium for clearing. It manifests somewhat the same qualities as the dehydrant, being neither volatile, hygroscopic, nor markedly inflammable, and having no shrinking, distorting, nor hardening action on the tissues. The

clearing agent removes all dehydrant, at the same time so conditioning the tissues that they will cut easily. Highly soluble in paraffin, it also renders the tissues translucent, increasing their refractive index.

From 6 A.M. to 7 A.M. the cleared tissues enter and remain in the first paraffin bath. From 7 A.M. to 9 A.M. they undergo a second paraffin immersion. It is always well to employ two changes of paraffin. While the first becomes contaminated, the second stays reasonably free of contaminants. Paraffin used in infiltrating the tissues should have a relatively low melting point (54° to 58° C.), so that tissues are not subjected to high heat for prolonged periods.

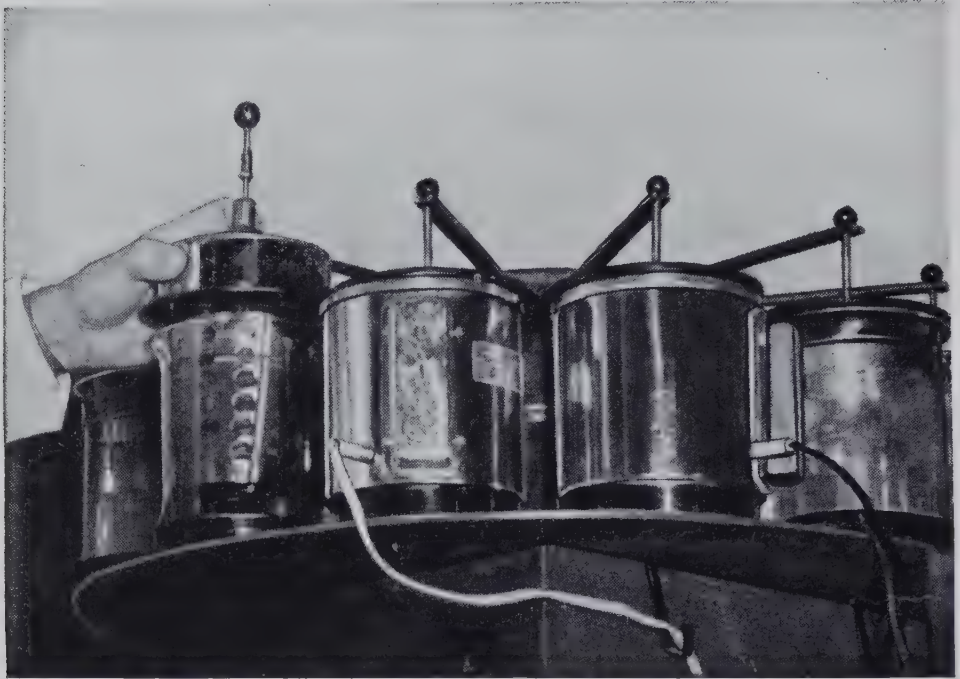


Fig. 70.—With the machine now in operation, the tissues are moved from fluid to fluid in accordance with the predetermined plan.

At 9 A.M. (or whenever the working day starts), the laboratory technician arrives. He lifts the paraffin bath with its contained load of tissues in the receptacle basket, and carries it to the embedding bench. Here, he plugs the bath into a convenient electric outlet to maintain the paraffin at the desired fluidity.

The tissue receptacles are now removed one by one from the paraffin. A receptacle is laid on the table, bottom down. With the forceps handle, the top is struck lightly, causing the clamping lugs to spring open with a snap that breaks loose any adhering paraffin.



Fig. 71.—Tissues are removed from their receptacles with forceps, and placed in a flat pan of melted paraffin.



Fig. 72.—Rubbing ice along the bottom of the pan causes the paraffin to solidify locally, anchoring the tissues securely in place.

Now, with the same forceps, the tissues are removed from the receptacle (each with its identifying label) and placed in a flat pan of melted paraffin. For embedding purposes, it is recommended that a paraffin of a slightly higher melting point be used (i.e., 60° to 62° C.). A Bunsen burner, or similar heating device, is placed beneath the pan to keep the paraffin fluid during embedding. It is convenient to set the pan on a fairly high stand so that ice may be rubbed along the bottom. This causes the paraffin to solidify locally, anchoring the tissues securely in place. The pan should contain about half an inch of paraffin, the tissues embedded with the

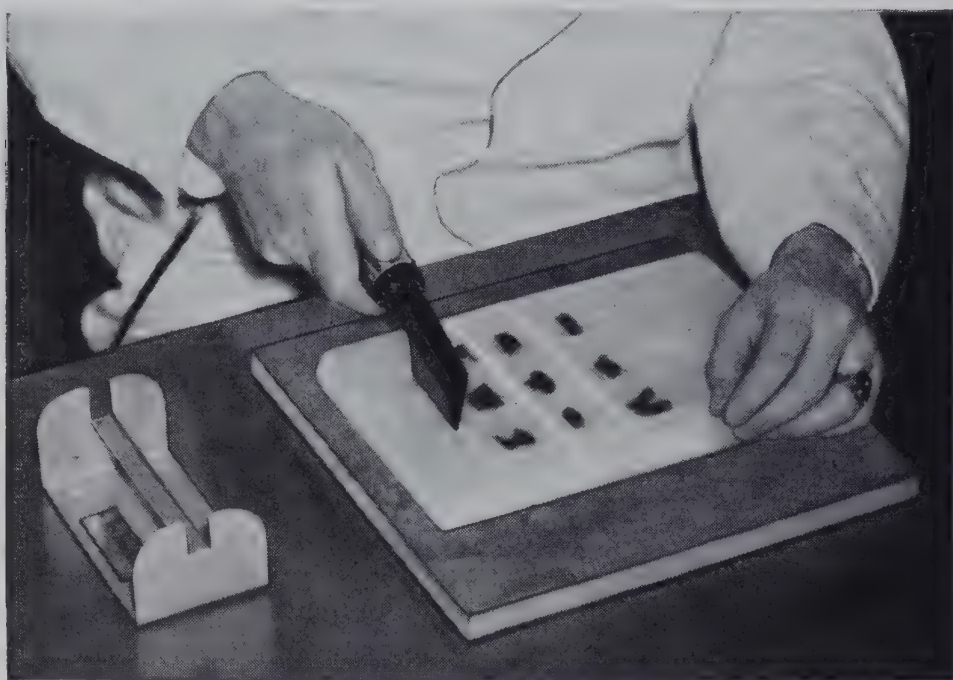


Fig. 73.—Here the technician scores the large paraffin block so that it may be broken into individual small blocks.

side to be cut facing down. Before pouring in the paraffin, the pan should first be lightly coated with glycerin so that the final paraffin slab can be easily removed when embedding is completed. Tissues should be placed in straight rows so that neatly squared blocks can be cut when the paraffin slab is divided for sectioning. To avoid crystalline formations, the slab should cool slowly. It is best to leave it standing at room temperature until it congeals. Once congealed, the paraffin slab should be cooled in water for hardening. Avoid water of too low a temperature, as it will tend to form crystals, making the tissues difficult to cut.

When the hardened slab is freed from the pan, it is cut into individual blocks, each containing a piece of tissue with its accompanying label. This is easily done with a Technicon paraffin knife, whose hot edge scores the slab deeply so that it is easily broken into individual blocks. Once these rough blocks have been obtained, the hot knife is again used for final trimming. An easy way to do this is to place the side of the knife against the side of the paraffin block. The paraffin starts to melt; thus it is a simple matter to obtain a square block.



Fig. 74.—Final trimming of the rough blocks is easily accomplished by placing the side of the Technicon paraffin knife against them.

The next step is to cut the tissues on the microtome. A single block is taken and its label removed with the heated paraffin knife. The block must now be mounted on the microtome object disk. An easy method is to place the stem of the object disk in a narrow-necked bottle to support it upright. Hold one side of the heated knife blade against the face of the disk, while holding the block against the other. Slide the knife out from under, and the block will stick to the disk. Next, the identifying label is replaced, the object disk is inserted in the microtome, and cutting is started. It is important that the knife angle be correct. For best results with most tissues there should be a 5 degree angle between the side of the

paraffin block and the bevel of the microtome knife. A convenient way to determine this angle is to put the sharpening back on the knife, then hold a straight edge between the back and the edge of the knife to determine the exact angle, which should be approximately 5 degrees. In cutting, avoid taking a thick preliminary trimming cut. If very thick cuts are taken for rough trimmings, the knife edge is rapidly dulled. (Always put the narrower side of the tissue block nearest the knife when inserting the block into the microtome.)

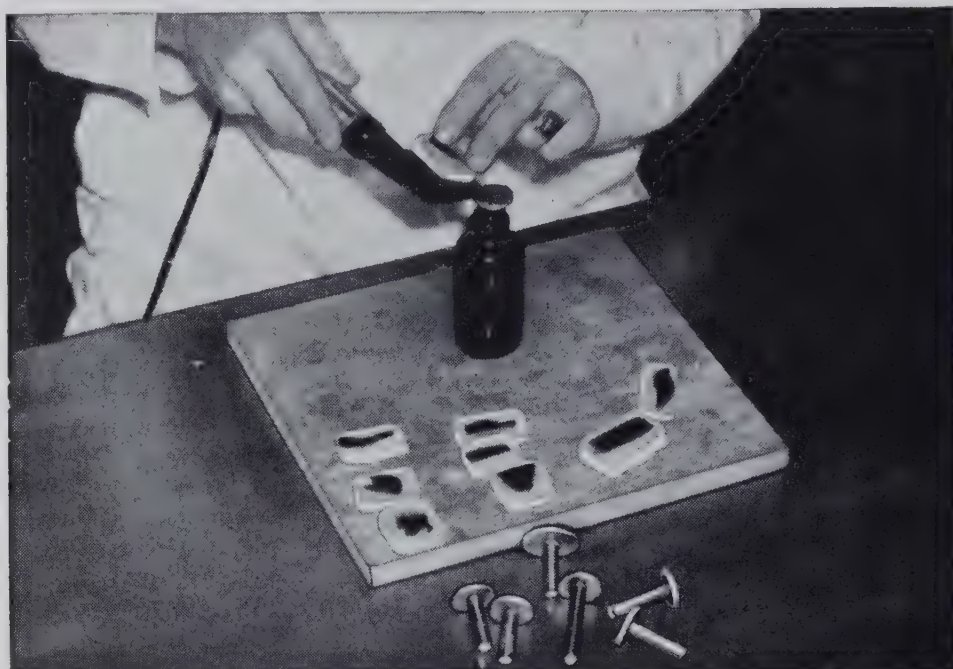


Fig. 75.—With the stem of the object disk in a narrow-necked bottle, the block is slid off the paraffin knife onto the face of the disk.

As the cut tissues are lifted from the microtome, they are spread face up on a Technicon constant temperature water bath. Before a cut tissue section is picked up and placed on a slide, the number on the identifying label is scratched on the slide with a diamond point (a vibrating hand tool will etch the glass very efficiently, if one is available). The thermostat on the water bath should be set at 45° C. for the best results and the level of the water in the bath should be very near the top. The 45° temperature so flattens the tissues that they will adhere properly to the slide when mounted. Unwanted tissues still floating in the bath are removed by dropping a paper towel flat on the surface. When it is lifted, the paraffin ribbons will come away with it.

To mount the tissues, the technician places a small amount of egg albumen on a slide to serve as an adhesive. The slide is then dipped under the floating ribbon; as it is lifted, the tissue adheres flat and secure. As an extra precaution, at this point some technicians make it a practice to *quickly* dip the mounted slide in a container of very hot water. The quick dip completely flattens the tissue section by barely melting the surface paraffin for closer adhesion. Water at approximately 95° C. is good for this purpose.

As the slides are mounted, they are inserted in the staining rack which is then placed in a Technicon microslide dryer. Here, a current of warm, filtered air (56° C.) passes over them, completely drying the slides in about 7 minutes.

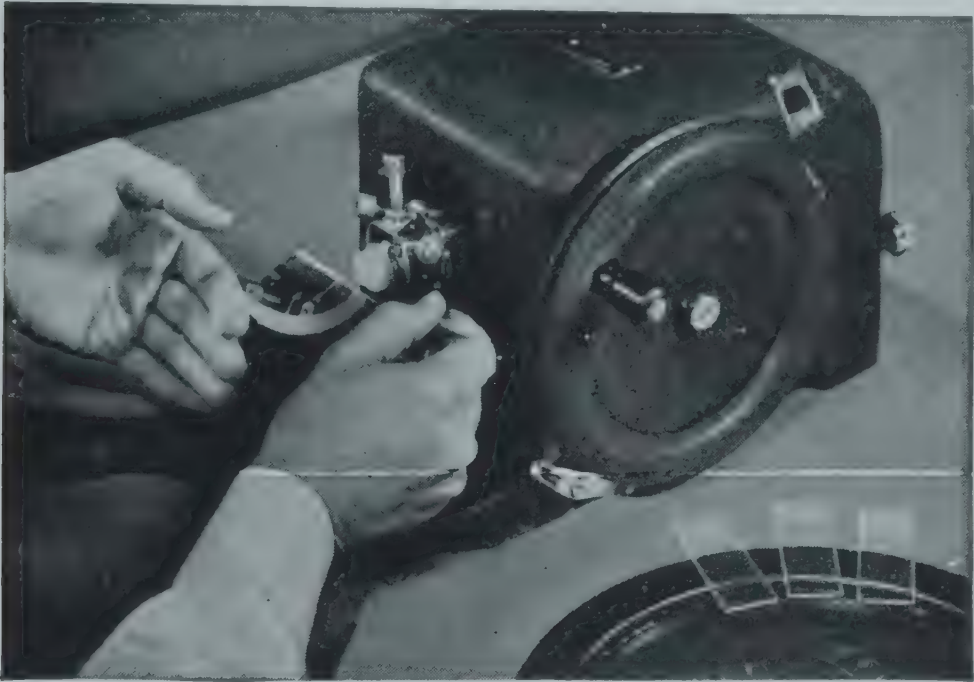


Fig. 76.—Cut tissues are lifted carefully from the microtome and spread face up on the Technicon constant temperature water bath.

The dried slides, still in the rack, are now taken from the drier, and the rack mounted on the Autotechnicon for staining. As in the earlier processing steps, operation from this point is completely automatic, and may proceed while the technician takes lunch, or goes about other duties.

The tissues now move first into a beaker containing xylene, which removes paraffin during a 2-minute immersion period. Next, they go into 0.25 per cent iodine dissolved in the dehydrant, which will

remove any mercuric chloride crystals persisting in the tissue. Here they remain for 2 minutes. The next step (a bath of straight Technicon dehydrant) removes excess iodine in two minutes. The fourth step is distilled water for 2 minutes more. The slides are now ready for staining in hematoxylin. A formula offering many advantages in this staining step is Technicon standardized hematoxylin. Ten minutes, more or less, completes this first staining; the time varies according to the intensity of color required. Technicon standardized hematoxylin is a delicately balanced, stable dye absolutely selective in staining action. It will act upon basophilic

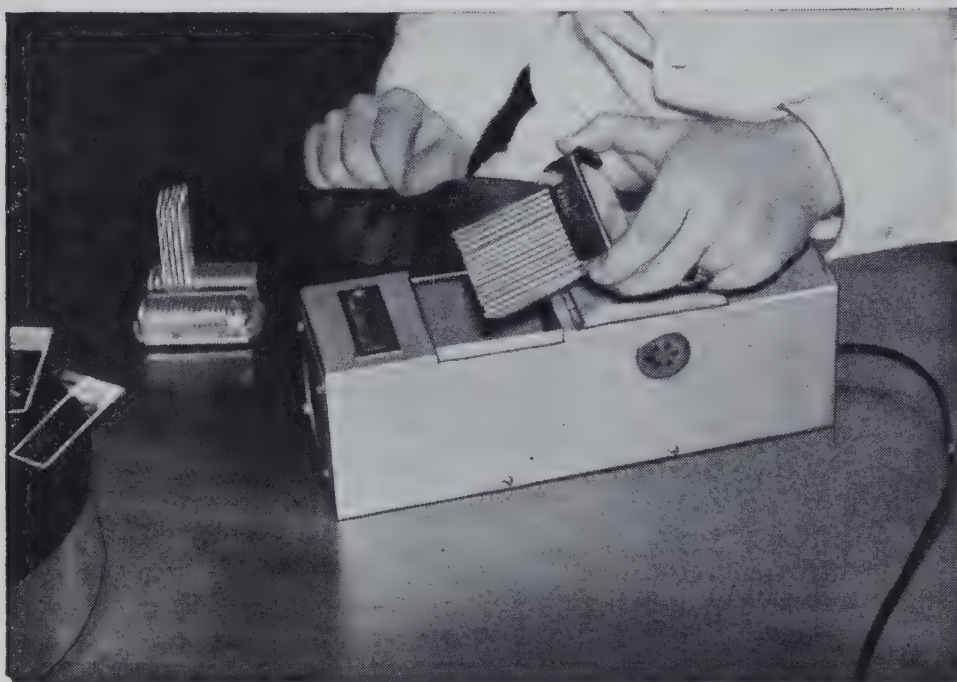


Fig. 77.—Mounted slides are inserted in the staining rack, which is then placed in the Technicon microslide dryer.

components only, rejecting acidophilic cells. This standardized hematoxylin is so selective that no subsequent decolorization is necessary. Stain penetration is progressive, the intensity of color being proportional to the length of immersion. After this first staining, the next step is a rinse of distilled water for 4 minutes to remove excess hematoxylin. The seventh place is lithium carbonate, which renders the hematoxylin alkaline, making the dye blue for better contrast. Technicon tablets of lithium carbonate are very convenient here; one tablet dissolved in one liter of distilled water gives a 0.01 normal solution. Tissues stay in this solution for

2 minutes. Then they move into the eighth place of distilled water for 2 minutes more to remove excess lithium carbonate. (Lithium carbonate carried over to the next step, staining with eosin, would have an adverse effect.)

The ninth place is 5 minutes in eosin. Here again, the special standardized eosin formula prepared by Technicon has many virtues not found in ordinary eosin solutions. Like Technicon-standardized hematoxylin, it will last, with steady use, for approximately six months, staining qualities remaining uniform over the entire period. At the end of its useful life, the solution goes sour and staining efficiency sinks to zero as the balance is destroyed. No subsequent decolorization is required when Technicon eosin is employed. This standardized eosin stains only acidophilic material; it has no effect whatever upon basophilic cells.

The tenth place is distilled water to remove the excess eosin adhering to the slide.

Steps eleven and twelve are 5 minutes each in the dehydrant, in order to dehydrate the tissues so that they may be placed in xylene without clouding. (Residual water in the tissues tends to cloud the xylene and the resultant slides lack clarity.) After dehydration, the tissues are removed and placed in xylene. Using tongs, the staining rack with its load of stained slides is placed upside down in a beaker of xylene.

Slides are now removed from the rack, singly. The slides are cleaned and fitted with cover glasses. Many technicians find it easier to apply cover glass by reversing the usual procedure; that is, by placing the slide on the cover glass, rather than vice versa. Place the cover glass flat on the table; apply a drop of adhesive to it; then carefully lay the slide upon it. In this way, the larger slide is handled instead of the small cover glass, making the operation much simpler.

A transparent plastic adhesive is available for adhering cover glasses; it does not discolor with age like Canada balsam. Canada balsam also has the disadvantage of becoming acidic when slides treated with it are projected, thus causing rapid fading. It also dries very slowly, often staying sticky for years before drying completely.

When the cover glasses are finally attached, the process is completed and the tissues are ready for microscopic examination.



Fig. 78.—The cover glass is placed flat on the table; a drop of adhesive is applied and the slide then carefully laid upon it.

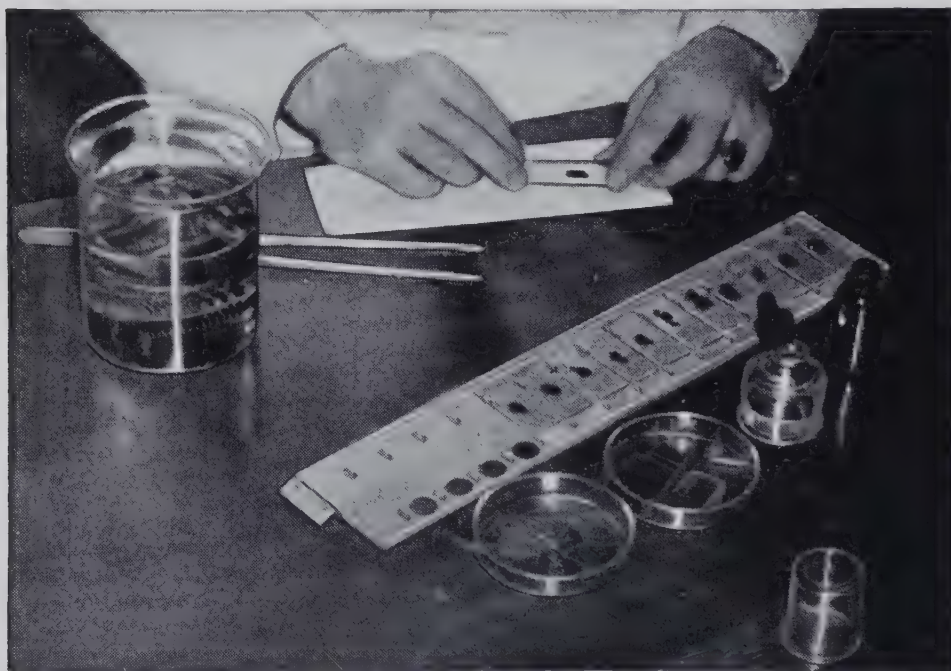


Fig. 79.—With the tissues now ready for examination, the slides are placed in a Technicon flat-filing tray and taken to the pathologist.

The slides are placed in a Technicon flat-filing tray, and brought to the pathologist for diagnosis. A microphone setup, similar to that mentioned earlier, is convenient for describing the sections. On the completion of microscopic examination, the slide-filled tray is placed in a flat-filing cabinet, which holds fifty similar trays, carrying sixteen slides each. Because slides do not contact each other in this cabinet, and air can circulate around them freely, they dry rapidly for early removal to a microslide filing cabinet. Here they are kept vertical in narrow drawers with a unique system of spring spaces which makes it easy to find, insert, or remove slides. After

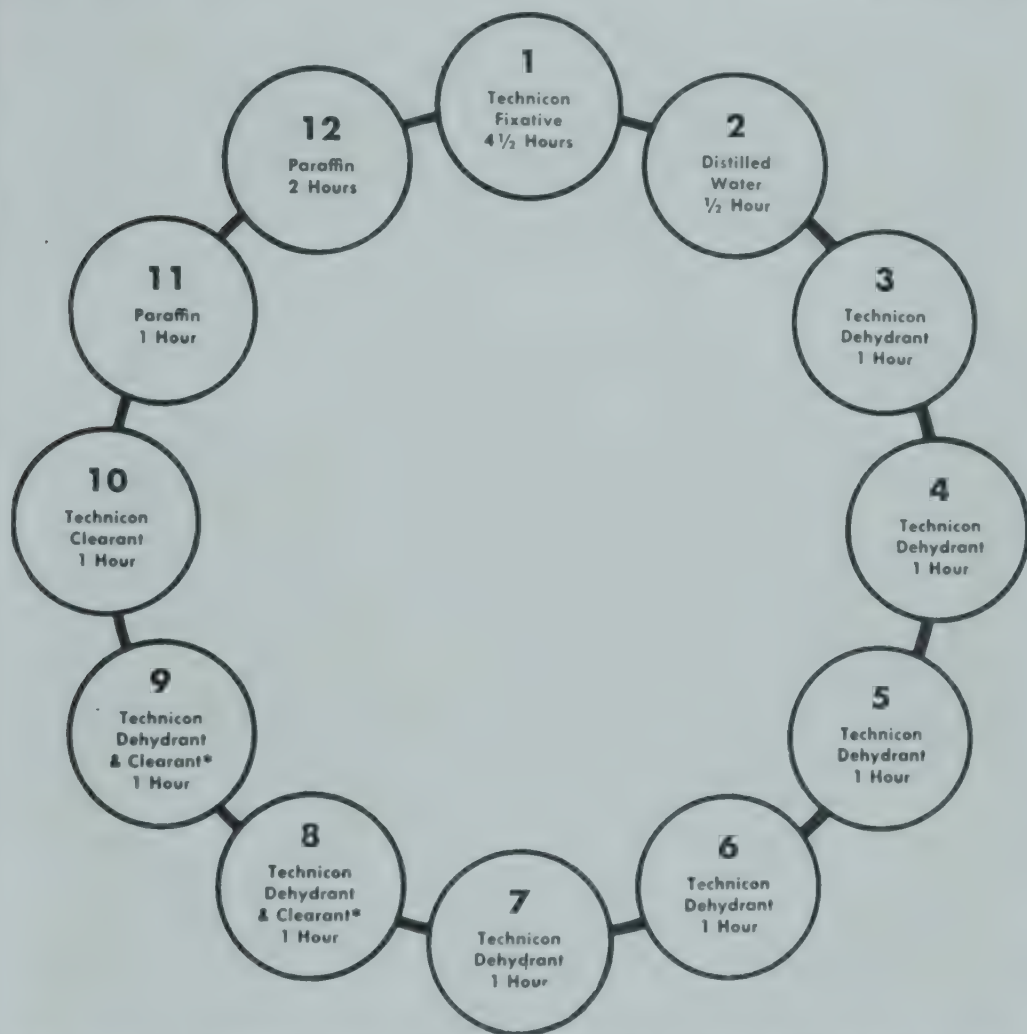


Fig. 80.—A representation of Autotechnicon's twelve stages set up for the fixation and dehydration phase of histological processing. In each circle is shown the reagent used and the immersion time for which Autotechnicon is set for that stage. This process is entirely automatic with Autotechnicon, which advances the tissues from stage to stage at set intervals and, when the cycle is completed, stops. The usual procedure is to start fixation at 5 p.m. The Autotechnicon will then have the tissues prepared, ready for blocking at 9:30 a.m., having carried on unattended all through the night.

*Equal parts of Technicon dehydrant and clearant.

a six-month period, when the slides are thoroughly dry and when the likelihood of requiring them for quick reference has gone, they are removed from the current space filing cabinet to close-packed storage in microslide cabinets for permanent storage.

For preserving an orderly arrangement, the paraffin blocks are filed away in paper boxes. These boxes are uniform in width and height, but vary in length, so that single blocks or multiple blocks, surgical cases, or autopsies may all be accommodated, each in a single block box.



Fig. 81.—Autotechnicon's twelve stages set up for staining. In each circle is shown the reagent used and the immersion time for which Autotechnicon is set for that solution. Staining is also entirely automatic with the Autotechnicon, which advances the slides from stage to stage and, when the cycle is completed, stops. Staining is performed completely automatically by the Autotechnicon while the technicians busy themselves with other duties. When the staining cycle is completed, the sections are ready for clearing in xylene and for application of the cover glasses.

**An approximately 0.01 normal solution is prepared by dissolving 1 Technicon lithium carbonate tablet in a liter of distilled water.

***After the twelfth stage, slides are transferred to a beaker of xylene. After 3 minutes' immersion they may be put in another change of xylene, from which the cover glasses are applied.

Faults in Dehydration, Clearing, and Embedding, and the Remedies

If the tissues are insufficiently dehydrated, they cause the clearing reagent to turn milky. Thus, to correct this, dehydration with absolute alcohol should be repeated.

If the tissues are insufficiently cleared, they become opaque and section-cutting is impossible owing to the presence of alcohol. Repeat the process of clearing, leaving the tissues in the clearing reagent for 2 hours.

The room temperature, the condition, and the position of the knife and paraffin block markedly affect the cutting of good sections. The morning is preferable, as the temperature is more favorable. It is essential to have a basin containing chipped ice ready to keep the paraffin blocks cool.

In cutting paraffin sections, sometimes the ribbons are curved instead of straight. This is caused by lack of parallelism of the surfaces of the blocks and may be remedied by unscrewing the block holder and trimming the block properly.

Sometimes sections are cut well individually, but do not adhere to one another to form a ribbon. This is due to the block being too cold. Warming the knife over the flame or in warm water corrects this condition.

Crumbling of the sections is caused either by the paraffin being too soft or by the knife being too dull. This may be prevented by cooling the block in ice water and sharpening the knife by stropping.

Irregular cutting of the sections, sometimes thick and sometimes thin, is caused by improper adjustment of the knife or block holder and may be corrected by tightening the screws of the block holder and the knife. If scratching of the section occurs as a result of nicks on the knife, the nicks should be removed by honing.

Celloidin Embedding and Sectioning Methods

After they are properly fixed in any given fixative, tissues are subjected to one of the three celloidin embedding processes described below:

Wet Celloidin Embedding Process

Tissue blocks, not more than 2 to 3 mm. thick, are hardened and dehydrated for 24 hours in 80 per cent alcohol, followed by 24

hours in 95 per cent, and finally in absolute alcohol for an equal period. They are soaked in a mixture of equal parts of absolute alcohol and ether for from 24 to 72 hours, and are then first impregnated in medium celloidin solution for from 2 to 4 days, sometimes longer, depending on the thickness and consistency of the tissue. Finally, they are soaked in thick celloidin for from 3 to 6 days. The blocks are then embedded in paper boxes or are mounted on vulcanized fiber.

To harden the celloidin, place the block in a desiccator jar and lift the cover once or twice a day for a moment to allow evaporation and thickening of celloidin. When it attains the right consistency, add several drops of chloroform to the bottom of the desiccator. The chloroform vapor hardens the celloidin, a process requiring several hours for small objects to several days for large ones. Then place the celloidin blocks in 80 per cent alcohol.

Steps of celloidin embedding process:

1. Harden and dehydrate in 80 per cent alcohol for 24 hours.
2. Repeat in 95 per cent alcohol for 24 hours.
3. Repeat in absolute alcohol for 24 hours.
4. Soak in equal parts of absolute alcohol and ether for from 24 to 72 hours.
5. Impregnate in medium celloidin for 2 to 4 days, or longer, depending on the thickness and consistency of the tissue.
6. Soak in thick celloidin for 3 to 6 days.
7. Embed in paper box or mount on vulcanized fiber.
8. Harden in a desiccator with chloroform vapor, and place in 80 per cent alcohol.

To mount, trim the block to proper size, dip the base in thick celloidin, press it firmly on vulcanized fiber, and treat with chloroform vapor as described above.

Keep the block in 80 per cent alcohol until it is sectioned.

Celloidin is a purified form of collodion or nitrocellulose. For histological work this is supplied in small quantities (ounce) in brown bottles.

Advantage of the Celloidin Method:

1. For large tissues, long period of immersion in celloidin does not affect the consistency. Such objects treated by the paraffin method become very hard, since prolonged infiltration is necessary to insure complete penetration.

2. For hard objects, such as eye and bone tissue, celloidin provides better support than paraffin and does not crumble in sectioning.

Disadvantages:

1. Very thin sections cannot be obtained.
2. Embedding process is very slow.
3. Serial sections are difficult to prepare.

Dry Celloidin Embedding Process

Follow the wet celloidin embedding process and mount the tissue blocks on vulcanized fiber. Expose them to the air until the celloidin mass attains the right consistency, then instead of hardening in chloroform, place them in Gilson's mixture, made up of equal parts of chloroform and cedar oil. Add cedar oil at intervals of one hour for small blocks and 10 hours or longer for larger blocks, until the mixture is about 90 per cent cedar oil. By this time the celloidin becomes very transparent. Then expose the blocks to the air until dry.

The blocks are kept in stoppered bottles and are cut dry.

Celloidin-Paraffin Embedding Process

The following technic enables one to obtain serial sections by embedding celloidin blocks in paraffin mass:

Embed blocks in celloidin by the dry process. Trim the celloidin blocks and transfer them from cedar oil into melted paraffin in an oven at 55° C. for about 2 hours. Place the blocks in paper boxes containing fresh paraffin, and harden by placing on a wet towel.

For cutting, follow the paraffin sectioning method.

Celloidin Sectioning Method

The celloidin method is used for large objects and for hard and delicate material. The celloidin is not removed from the tissues and holds the delicate structures together.

The material to be cut is embedded in one of the commercial celloidins (cellulose nitrate), the slow burning kind being preferred. One of the disadvantages of the method is the longer embedding time necessary. Another is that serial sections cannot be properly cut, so that each section has to be handled individually.

For materials not injured by moderate amount of heat, the rapid method facilitates impregnation and preparation of the tissue. The slower, cold process takes more time and does not damage the tissue.

The chief difficulty in celloidin sectioning arises from trying to cut improperly fixed material. Inadequately hardened blocks cannot be sectioned successfully. The blocks should be hard enough to cut at the required thickness. Unless the block is sufficiently hardened, the sections are uneven and distorted. If the block is too hard, irregularities are likely to occur. The surface of the block and the surface of the knife should be kept wet with 70 per cent alcohol, and as soon as sections are removed they should be placed in alcohol.

The sliding microtome is the instrument of choice for cutting celloidin sections, using a slice angle of 10 to 40 degrees. Tilt the knife a little more than for cutting with paraffin, though the actual tilt will depend upon the hardness of the tissue. It is convenient to let large sections roll up on the knife; then lift off the knife and unroll into 70 per cent alcohol. Or remove sections from the knife and lay a piece of filter paper on top of the section. It usually adheres to the filter paper and then both may be placed in a Stender dish. If the filter papers are numbered they may be kept in order. Or, stamp a number on the margin of the section with a commercial numbering machine as each one is cut.

The knife for sectioning celloidin-embedded material should be very sharp. Any irregularities in the edge will leave marks on the section. Keep the section and materials free from dust. Dust particles catching on the edge of the knife spoil the section, particularly when very thin sections are required. Decalcify first if any calcium is present in tissue. Do not allow the surface to dry. If it is necessary to stop sectioning for even a short period, cover the block with absorbent cotton saturated with alcohol, or remove the block and place in 70 per cent alcohol.

The so-called dry process has been developed to replace the method which requires keeping the block and knife wet with alcohol. After the tissue is embedded and hardened, soak the block in an oil (cedar oil) which lubricates the block at the time of cutting. As the sections are removed from the knife, place them in the same oil. The oil-soaked block may be cut on a rotary microtome, as no slicing cut is required. This method is satisfactory for making sections of difficult organs.

Special knife holders are available so that the knife can be turned to give a slicing stroke and may be used for cutting small celloidin blocks on a rotary microtome. The position of the block and knife is such, however, that it is difficult to keep the surfaces properly lubricated with alcohol so that the rotary microtome is not very satisfactory for this type of sectioning. If much sectioning work in celloidin must be done on a rotary microtome, it is advisable to use the dry method which does not require a slicing stroke.

Celloidin-embedded blocks are sometimes embedded in paraffin to hold them for sectioning. The size of the section which may be cut depends on the size of the microtome and the knife and the skill of the operator.

Celloidin sections are usually stained in Stender dishes or watch glasses. They are not fixed to the slides until they are ready for mounting. Sometimes it is more convenient to stain them on the slide by the following method:

The sections are transferred from water to the slide and are dried by blotting in fine filter paper. Next they are covered with absolute alcohol for a half minute and again blotted. The sections are then dipped in thin celloidin, the excess quickly drained off. The breath is blown briskly over them and they are immersed in water.

Celloidin sections can be stained by nearly all methods without removing the celloidin. When it is necessary to remove the celloidin, the sections are placed in oil of cloves or ether-alcohol mixture for 5 to 10 minutes, and then passed through absolute alcohol in 95 per cent alcohol.

During the process of staining, most celloidin sections swell and shrink. To flatten them, treat in equal parts of absolute alcohol and oil of cloves for 2 or 3 minutes before placing in xylol.

The two solutions spoken of as *thick* and *medium celloidin* are prepared as follows:

Thick Celloidin.—Thirty grams of dry celloidin are dissolved in 200 c.c. of a mixture of equal parts of ether and absolute alcohol. Shake the receptacle several times a day for one week or longer, and stir the sediment with a heavy glass rod at least once a day until solution is complete.

Medium Celloidin.—Medium celloidin is made by diluting thick celloidin with an equal amount of ether and alcohol mixture.

The thin celloidin used in attaching sections to the slide is made up of 5 to 8 c.c. of thick celloidin in 95 c.c. of ether-alcohol-acetone mixture:

Ether	-----	45 c.c.
Alcohol, absolute	-----	45 c.c.
Acetone, C.P.	-----	10 c.c.

If the mixture is too thick, add more ether-alcohol-acetone solution; if too thin, add more thick celloidin. Schering's or Du Pont's celloidin is preferable. It slowly deteriorates and must be used within one year after the date of manufacture.

The addition of 1 c.c. of saturated alcoholic solution of gum mastic to thin celloidin strengthens the adhesive property, makes sections smooth, and prevents rapid deterioration (Krajian).

Difficulties in Handling Celloidin-Embedded Material

1. The chief difficulty comes from improperly prepared material. Adequate impregnation of a large organ like a hemisphere of a brain may take a year. Improperly hardened blocks cannot be sectioned successfully.

2. Lengthwise scratches or splits in the section may be due to:

a. Nicks in the knife. Use a different part of the knife or re-sharpen.

b. Particles of hard material in the block. If there is dust or dirt in the celloidin stock solution, let solution stand and use only upper portion after the particles have settled out, or filter the stock solution. If calcareous deposits are present, decalcify.

3. Specimen falls out of section, is mushy and soft.

a. Dehydration was incomplete.

b. Infiltration was incomplete—refilter, re-embed, and harden.

c. Harden block, if too soft, in chloroform, or a mixture of equal parts of 95 per cent alcohol and glycerin.

4. Variation in thickness of sections.

a. Loose screws on knife or block holders—tighten all set screws.

b. Knife holder depressed or raised by the hand while sectioning—hold knife block so as not to move it vertically while cutting.

c. Knife not tilted enough to clear facet of cutting bevel.

d. Knife too dull.

e. Microtome worn and out of adjustment.

f. Material not hardened properly.

g. Slight drying of the block between sections.

PART IV

STAINING SOLUTIONS

Hematoxylin Stains

Hematoxylin is one of the oldest and most valuable histological stains. Hematein, the active coloring agent, is formed by oxidation, a process requiring a number of days or weeks.

In alum hematoxylin, the selective staining power is due to the combination of hematein with alum. Mayer, Unna, and Harris have shown that it is possible to oxidize and ripen the alum hematoxylin by adding a little mercuric oxide or hydrogen peroxide.

As alum hematoxylin becomes older, they stain more quickly. The addition of 3 c.c. glacial acetic acid to 95 c.c. hematoxylin solution at the time of use helps to sharpen the nuclear staining of the cells.

Delafield's Hematoxylin

Hematoxylin crystals	4 Gm.
Alcohol, 95 per cent	25 c.c.
Saturated aqueous solution of ammonium alum	400 c.c.

Dissolve the hematoxylin crystals in alcohol and add to the alum solution. Expose the mixture in an unstoppered bottle to light and air for 10 days.

Filter and add:

Glycerin	100 c.c.
Alcohol, 95 per cent	100 c.c.

Allow the solution to stand in light until the color becomes a dark purple, then filter and keep in a tightly stoppered bottle. The solution keeps well and is very powerful as long as it has a purplish tinge.

Harris' Hematoxylin

Hematoxylin crystals	1 Gm.
Alcohol, 95 per cent	10 c.c.
Ammonium or potassium alum	20 Gm.
Distilled water	200 c.c.

Dissolve the hematoxylin in alcohol. Dissolve the alum in water by the application of heat, and add the hematoxylin solution. Bring

the mixture to a boil as quickly as possible, and then add 0.5 Gm. mercuric oxide. The solution at once assumes a dark purple color. As soon as this occurs, remove the vessel containing the solution from the flame and cool as rapidly as possible in a bath of running, or frequently changed, cold water. When cool, the solution is ready for use. Addition of 5 c.c. glacial acetic acid to 95 c.c. of solution at the time of use will increase its nuclear staining properties.

Harris' hematoxylin has been found very stable and useful in our tissue laboratories and is used routinely for all histological microscopic sections.

Mallory's Phosphotungstic Acid Hematoxylin

Hematein ammonium -----	0.1 Gm.
Water -----	100 c.c.
Phosphotungstic acid crystals (Merck) -----	2 Gm.

Dissolve the hematein in a small amount of water by the application of heat and add it, after it is cool, to the rest of the solution. If the solution stains weakly at first, it may be ripened by the addition of 5 c.c. of a 0.25 per cent aqueous solution of potassium permanganate, or it may be allowed to stand for several weeks until it ripens spontaneously.

Hematoxylin may be used instead of hematein ammonium, but requires 10 c.c. of permanganate solution to ripen it.

Mallory's phosphotungstic acid hematoxylin solution will be found especially useful to demonstrate fibrin (also the fibroglia and myoglia fibrils of neuroglia). It also brings out the detail of mitotic figures, including spindles and centrosomes.

Heidenhain's Iron Hematoxylin

Heidenhain's iron hematoxylin, after suitable fixation, is used to demonstrate chromatin, nuclei, nuclear membrane, and centrosomes. The fixatives generally used are chrome salts and corrosive sublimate. The staining solution is a 0.5 per cent ripened alcoholic solution of hematoxylin, and 4 per cent iron and ammonium alum.

CARMINE STAINS

The active staining principle in the carmine solutions is carminic acid.

All alkaline and acid solutions made with carmine owe their staining properties to carminic acid combined with aluminum.

Lithium Carmine

Carmine -----	2.5 Gm.
Lithium carbonate (saturated aqueous solution) 100	c.c.
Thymol -----	a crystal

The carmine dissolves at once in the cold solution. This solution should be carefully filtered, especially when used as a counterstain in the Gram-Weigert method.

Best's Carmine Stain for Glycogen*Stock Solution*

Carmine -----	2 Gm.
Potassium carbonate -----	1 Gm.
Potassium chloride -----	5 Gm.
Distilled water -----	60 c.c.

Boil several minutes, cool, and add:

Concentrated ammonia -----	20 c.c.
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Keep this stock solution in well-stoppered bottle in a dark cool place.

The Differentiator

Absolute methyl alcohol -----	40 c.c.
Absolute ethyl alcohol -----	80 c.c.
Distilled water -----	100 c.c.

Mayer's Mucicarmine

Carmine -----	1 Gm.
Aluminum chloride -----	0.5 Gm.
Distilled water -----	50 c.c.

Heat in a Pyrex beaker for several minutes until completely dissolved. Then add 50 c.c. of 95 per cent alcohol and mix (Krajian). The solution keeps well.

Carbolfuchsin Mixture*Mucin Stain (Krajian)*

Carbolfuchsin -----	15 drops
Acetic acid -----	3 drops
Formaldehyde, 40 per cent -----	3 drops
Water -----	2 c.c.

The solution does not keep, and therefore should be prepared fresh before use.

ANILINE DYES AND SOLUTIONS

Aniline dyes are sold in powder or crystal form and most of them keep well in that condition. It is well to keep on hand saturated alcoholic solutions of certain dyes, because they keep well in that form and are ready for use when wanted. This is particularly true of methylene blue, basic fuchsin, and methyl violet.

Aniline dyes are derived from either aniline or toluidine, or from both. They are salts having basic or acid properties. The basic colors stain the cell nuclei and bacteria. The acid colors stain diffusely.

The basic dyes are, chiefly, methylene blue, fuchsin, methyl violet, and safranine. The acid dyes most commonly used are eosin, picric acid, and fuchsin.

Unna's Polychrome Methylene Blue Solution

Methylene blue	-----	1 Gm.
Potassium carbonate	-----	1 Gm.
Water	-----	100 c.c.

The polychrome methylene blue solution of Unna is an old alkaline methylene blue. It requires months for the process of oxidation to take place at room temperature. The time is greatly shortened if heat is applied.

This stain has been extremely valuable as a stain for plasma cells and as a general stain combined with eosin, which should be used first. The methylene blue solution should be diluted 1:5 or 1:10 at the time of use.

Loeffler's Methylene Blue Solution

Saturated alcoholic solution of methylene blue	----	30 c.c.
Potassium hydroxide solution—1:10,000	-----	100 c.c.

This is a very useful aniline stain and keeps well for a long time without losing much power.

Pyronine-Methyl Green Stain (Unna)

Methyl green	-----	0.15 Gm.
Pyronine	-----	0.25 Gm.
Alcohol, 95 per cent	-----	2.5 c.c.
Glycerin	-----	20 c.c.
Carbolic acid water (0.5 per cent)	-----	100 c.c.

This solution is principally used in one of the chromatin methods.

Fuchsin (Basic) Solution

A saturated alcoholic solution of basic fuchsin should be kept in stock.

Basic fuchsin of superior quality, called "new fuchsin," is now on the market. It is highly recommended for its deep staining of acid-fast organisms.

Ziehl-Neelsen Carbolfuchsin

Saturated alcoholic solution of fuchsin	10 c.c.
5 per cent aqueous carbolic acid	90 c.c.

Make carbolic acid solution by adding 5 c.c. of melted carbolic acid to 95 c.c. of water. Shake and then mix with fuchsin and the solution is ready for use.

This solution is very powerful; it keeps and stains well. Shake the bottle before use.

Methyl Violet

A 1 per cent aqueous solution of methyl violet should be kept in stock. It keeps well and is used for staining bacteria and amyloid. It stains amyloid pink, but fades after mounting.

Gentian Violet

Gentian violet is a mixture of crystal violet, methyl violet, and dextrin. A product produced by various companies, called "gentian violet improved," has been found satisfactory.

Sterling's Gentian Violet

Gentian violet improved	5 Gm.
Alcohol, 95 per cent	10 c.c.
Aniline oil	2 c.c.
Distilled water	88 c.c.

Dissolve 2 c.c. aniline oil in 88 c.c. distilled water and filter through filter paper. Add to this the dissolved gentian violet in alcohol.

The solution keeps well from several weeks to months, and is highly recommended for demonstration of bacteria, fungi, and fibrin in tissue.

Eosin

Eosin is used principally as a counterstain after hematoxylin, and sometimes before methylene blue. It is sold in two forms as "eosin soluble in water" and as "eosin soluble in alcohol." The strength

to be used varies from 1 to 5 per cent. When eosin is used before an aniline dye, such as methylene blue, a 5 per cent solution should be taken. For counterstaining with hematoxylin, a 1 per cent aqueous solution has been found very satisfactory.

Eosinol

(Krajian)

Dissolve 5 Gm. of aqueous eosin in 10 c.c. of distilled water. Precipitate it by adding 10 c.c. of glacial acetic acid, 2 c.c. of concentrated hydrochloric acid, and mix with a glass rod. Incubate the resulting coagulum at 56° C. for from 12 to 16 hours, or until all the water has evaporated.

Dissolve this dehydrated acid eosin in 10 c.c. of absolute alcohol or isopropanol (anhydrous) and 20 c.c. of acetone, stirring with a glass rod for several minutes and placing in paraffin oven for 2 hours. Let the undissolved portion, which is to be discarded, settle to the bottom of the container. Remove the clear portion with a clean dry pipette; add it to 1,500 c.c. of carbolxylol (1 part pure phenol crystals in 3 parts of neutral xylol). Some precipitate will form, which will settle to the bottom of the container. The clear portion is eosinol. The solution keeps indefinitely.

Owing to variation in the staining power of the various brands of powdered eosin, it may be necessary to standardize the solution by staining control sections and adjusting the strength of the eosinol by reducing or increasing the amount of carbolxylol. (See method published in Arch. Path. **25**: 376, 1938.)

Phloxine

Phloxine has been in general use as a counterstain after hematoxylin, and some claim it to be superior to eosin. It stains more intensely than eosin, but differentiation is not as complete.

For counterstaining 1 per cent solution is taken.

✓ **Picric Acid Solution**

A saturated aqueous solution of picric acid is kept in stock to be used as needed. It is generally employed in van Gieson's stain and in Bouin's fluid.

✓ **Acid Fuchsin Solution**

A 1 per cent aqueous solution of acid fuchsin should be kept in stock. This is used in van Gieson's connective tissue stain.

Orcein Solution

Orcein is a vegetable dye and is used mainly for elastic tissue stain. It is soluble in alcohol and is employed in either neutral or acid alcoholic solutions.

Acid Polychrome Methylene Blue

(Goodpasture)

Methylene blue -----	1 Gm.
Potassium carbonate -----	1 Gm.
Distilled water -----	400 c.c.

Dissolve the ingredients and boil in a flask for 30 minutes. The methylene blue will be polychromed and most of it will precipitate. When the solution is cool, add 3 c.c. glacial acetic acid. Shake thoroughly until the precipitate is dissolved, and then boil gently for 5 minutes, or until the solution is concentrated to a volume of 200 c.c. Cool the solution in tap water. The stain can be used immediately. The solution keeps indefinitely.

Tincture of Iodine

(Alcoholic Solution)

Tincture of iodine is principally used to dissolve the mercury precipitates formed in tissues that are fixed in bichloride of mercury or other fluids containing bichloride, such as Zenker's fluid.

Lugol's Solution

Lugol's is a solution of iodine in water, containing potassium iodide in varying strengths. Iodine in this form is used as a test for glycogen, amyloid, and corpora amylacea.

Iodine -----	1 Gm.
Potassium iodide -----	2 Gm.
Water -----	100 c.c.

Gram's Iodine

Iodine -----	1 Gm.
Potassium iodide -----	2 Gm.
Water -----	300 c.c.

Gram's iodine solution is used in the Gram-Weigert method of staining microorganisms and fibrin in tissue sections.

Acid Alcohol

Acid alcohol for destaining sections is prepared by mixing 1 c.c. concentrated hydrochloric acid with 99 c.c. of 70 per cent alcohol.

Ammonia Water

Ammonia water to blue sections is prepared by mixing 2 c.c. strong ammonium hydroxide with 98 c.c. tap water.

Sodium Thiosulfate Solution (Hypo)

The sodium thiosulfate solution, which is used to remove the color of iodine from sections, is prepared by dissolving sodium thiosulfate in alcohol and water.

Sodium thiosulfate -----	0.75	Gm.
Alcohol, 95 per cent -----	10	c.c.
Water -----	90	c.c.

Congo Red Solution

This is a 4 per cent aqueous solution of Congo red, used in the Krajan method of staining elastic tissue, amyloid, and myelin.

Silver Nitrate

(10 Per Cent Aqueous Solution)

A stock solution of silver nitrate is kept on hand to prepare various dilutions to be used in spirochete, reticulum, and numerous nerve fiber stains. Keep in dark place.

Developing Solution for Spirochete Stain

(Krajan)

The solution :

Hydroquinone -----	0.31	Gm.
Sodium sulfite -----	0.06	Gm.
Formaldehyde, 40 per cent -----	2.5	c.c.
Pyridine -----	2.5	c.c.
Acetone -----	2.5	c.c.
Gum mastic (saturated alcoholic solution) ----	2.5	c.c.
Distilled water -----	15	c.c.

Hydroquinone and sodium sulfite should first be completely dissolved in formaldehyde and acetone, then other reagents added and mixed, and finally the gum mastic solution. With the addition of the water, the mixture assumes a smooth creamy appearance.

Gum Mastic

(Saturated Alcoholic Solution)

Mastic solution is prepared by saturating 25 Gm. gum mastic in 35 c.c. absolute alcohol. Shake the solution several times a day for 3 to 5 days until it is clear. It is not necessary to filter; use only the clear portion.

Scharlach R (Scarlet Red) Solution Modification

(Krajian)

Scharlach R solution is prepared by saturating the dye (scharlach R) in

2 per cent benzoic acid (in 70 per cent alcohol) --	1 part
Acetone -----	1 part

The solution is very stable, and after being used may be poured back into the bottle and used over and over. Filter before use.

The addition of benzoic acid intensifies the staining property of fat and prevents rapid deterioration of the solution.

Giemsa's Stain

The Giemsa stain is valuable to demonstrate the following elements in the smear: red corpuscles, bluish; nuclei of leucocytes, purple; mast cells, purple; cytoplasm of lymphocytes, dark blue; parasites and bacteria, blue.

Azur II eosin -----	3.0 Gm.
Azur II -----	0.8 Gm.
Glycerin (Merck, C.P.) -----	125 c.c.
Methyl alcohol (acetone free) -----	375 c.c.

The solution will keep for many months in a well-stoppered bottle. This is a stock solution and must be diluted immediately before use.*

Weigert's Elastic Tissue Stain

(Modified)

A modification of the original Weigert method, Weigert's elastic tissue stain, gives better results and keeps longer in stock solution.

Mix and dissolve 2 c.c. carbolic acid and 1 Gm. basic fuchsin in 100 c.c. of distilled water, and bring the mixture to a boil. While boiling, gradually add 12.5 c.c. of 30 per cent solution of ferric

*Reliable Giemsa stain may be obtained from Gradiwohl Laboratories, 2514 Lucas Ave., St. Louis 3, Mo. It is Commissioned Certified.

chloride. A precipitate forms, and to render the precipitate more granular, continue boiling at least 5 minutes. Cool and filter. Wash the precipitate on filter paper with distilled water until no more color is seen.

Dry the precipitate in the paraffin oven at 55° C., and keep this dry powder in a bottle for making up staining solution when needed.

To make the staining solution, take 0.75 Gm. of the dry powdered precipitate, dissolve in 100 c.c. of 95 per cent alcohol, and boil in a flask plugged with cotton wool in a water bath for 20 minutes. Then cool, filter, and add 2 c.c. of concentrated hydrochloric acid. This is a valuable stain to demonstrate elastic fibers.

✓ **MacCallum's Elastic Fiber Stain**

(A Modification of Verhoeff's Method)

Hematoxylin, 10 per cent in absolute alcohol (the usual ripened solution) -----	2 c.c.
Ferric chloride, 10 per cent aqueous solution -----	3 c.c.
Sodium iodide, 10 per cent aqueous solution -----	5 c.c.
Alcohol, 50 per cent -----	50 c.c.

Mix above solution in the order given. Stain keeps about a week.

Krajan's Elastic Tissue Stain

(Modification of MacCallum's Method)

The solution:

Hematoxylin crystals -----	3 Gm.
Ferric chloride -----	3 Gm.
Ferric ammonium sulfate -----	3 Gm.
Potassium iodide -----	3 Gm.
Alcohol, 95 per cent -----	25 c.c.
Distilled water -----	25 c.c.

Dissolve the hematoxylin crystals in alcohol and the other reagents in water, and mix the two solutions together. The resulting solution stains elastic fibers and myelin sheaths blue black, acts as mordant in van Gieson stain, and stains bacteria.

✓ **Van Gieson Stain**

Van Gieson stain is a mixture of picric acid and acid fuchsin. It is used to demonstrate connective tissue fibers.

Picric acid (saturated aqueous sol.) -----	100 c.c.
Acid fuchsin (1 per cent aqueous sol.) -----	5 c.c.

The solution generally keeps well. If the stain weakens by long standing, its strength can be restored by the addition of a few drops of fresh acid fuchsin.

Acid Benzoic

(2 Per Cent Aqueous Solution)

The addition of alcoholic benzoic acid to scarlet red solution intensifies the fat stain and prevents deterioration of the staining solution (Krajian).

Sodium Cyanide

(2 Per Cent Aqueous Solution)

Sodium cyanide solution is used in fat and reticulum stains.

Primary Solution

The primary solution is used in staining spirochetes (Krajian).

Uranium nitrate -----	5 Gm.
Formic acid, 85 per cent -----	15 c.c.
Glycerin -----	25 c.c.
Acetone -----	50 c.c.
Alcohol, 95 per cent -----	50 c.c.

Dissolve uranium nitrate in formic acid, add other reagents, and mix.

The solution is permanent.

PART V

CLEARING REAGENTS

Clearing reagents are used to make the tissue sections transparent so that they may be seen and differentiated under the microscope.

For clearing the tissue sections, a variety of reagents of different chemical properties is used. The choice depends mainly on two factors: the kind of stain used, and the materials in which tissues have been fixed and embedded.

Most clearing reagents can be used after hematoxylin stain. For celloidin sections, oil origanum and oil of bergamot are recommended. For aniline stains, xylol is the best clearing reagent, but it clears only from absolute alcohol and butyl alcohol.

Carbolxylol has a dark straw color. It does not dissolve celloidin and it does not affect hematoxylin. It clears directly from absolute and butyl alcohols, and it will clear from 95 per cent alcohol also if the section is blotted in filter paper before it is placed in carbolxylol. Sections should not be left more than 5 minutes in this mixture.

Xylol is colorless. It does not dissolve celloidin nor does it affect aniline dyes. It clears directly from absolute or butyl alcohols, and it will also clear from 95 per cent alcohol if the xylol is used in several applications, the section blotted after each.

Oil origanum is straw to brown in color, and affects aniline stains slowly. It clears celloidin sections very slowly.

Oil of bergamot is light green in color, and clears from 95 per cent alcohol. It does not dissolve celloidin, and affects aniline stains slowly, except eosin, which it extracts very quickly.

Oil of cloves is straw colored. It clears from 95 per cent alcohol, affects aniline stains, especially methylene blue, and dissolves celloidin slowly.

Aniline oil is colorless when perfectly pure and fresh, but oxidizes rapidly and turns brown. It does not dissolve celloidin, clears from 80 per cent alcohol, and affects aniline stains slowly. A mixture of

equal parts of aniline oil and xylol is used to decolorize sections stained by the Gram-Weigert method, also in Flexner's stain for leprosy bacilli, and to clear sections stained for myelin sheath by the Courville-Krajian method.

Oil of cedarwood has a pale straw color. It clears from 95 per cent alcohol, does not affect aniline stains, and clears celloidin sections very slowly.

Beechwood creosote, formerly much used to clear celloidin sections, is replaced with carbolxylol.

Chloroform has long been used to clear tissues after dehydration. It absorbs a good deal of moisture left in tissues and renders them less brittle.

Benzine is too volatile for clearing purposes. Some workers prefer it to chloroform or xylol because it clears quickly, makes the tissue more transparent, and evaporates rapidly from paraffin bath.

PART VI

MOUNTING REAGENTS

For permanent mounts, the reagents in general use are gum dammar and Canada balsam.

We have been using gum dammar as a routine in the mounting of permanent microscopic tissue sections for a period of ten years. In this period we have prepared approximately 250,000 sections. This mounting medium has proved to be superior in every way to Canada balsam, which was formerly used. We believe an effort should be made to inform pathologists and laboratory workers of the advantages of gum dammar.

The possibilities of this material were first called to the attention of Dr. Newton Evans by Dr. Francis Carter Wood, who recommended its use for the routine preparation of histological specimens, saying he had used it exclusively for a number of years and found it entirely satisfactory.

In a recent comparative examination of old slides mounted in Canada balsam and others mounted with gum dammar, it was found gum dammar was superior.

1. The balsam slides are much dirtier and mussier in appearance.
2. They dry slowly and, after years of curing still have a tendency to stick together when stored in contact.
3. The balsam has turned yellow at the edges and under the cover slips.
4. The edges of the sections, within a zone 2 or 3 mm. wide, at the edge of the cover slip, are completely decolorized as far as the nuclear stain is concerned.

On the other hand, the slides mounted with gum dammar are much cleaner, dry more quickly, do not stick together if allowed to dry for a few months before storing in contact with other slides, do not discolor, and do not fade. An important advantage is the possibility of storing slides in compact arrangement, thus saving much space and expense in providing cabinets for the permanent filing of the slides.

Prepared solutions of gum dammar ready for use can be purchased from laboratory supply houses. Its satisfactory preparation is simple. The gum dammar is purchased in crude state from drug or supply houses and is dissolved in xylol.

Preparation.—Dissolve resin in the "neutral histological (practical) xylol," especially prepared by the Eastman Kodak Company, by placing ingredients together in a large stoppered bottle in an incubator, and stirring with a glass rod several times daily until the mixture has a heavy syrupy consistency. This usually requires about a week. Then strain through 4 layers of clean gauze and store in a dark bottle. If the resultant solution is too thin for mounting purposes, place the unstoppered bottle in an incubator and evaporate until it is of right consistency.

Canada balsam is prepared in the same manner as gum dammar.

Chloroform and Balsam. This is prepared by dissolving balsam in chloroform. The mixture is used as a mounting medium for tissue sections stained with osmic acid. Other mounting reagents used for osmic acid preparations fade the stain. The chloroform balsam mixture should be of heavy syrupy consistency.

Cedar oil is used to mount sections stained by Giemsa method. Only thick cedar oil, as that employed for microscopy, should be used.

Clarite is a resin dissolved in toluene and also in xylol. It is a better policy to purchase already prepared solution from supply houses.

Permout is prepared similar to clarite, and is supplied by Fisher Scientific Company and other supply houses.

It is a very satisfactory mounting reagent.

Colophonium is sold in solid form. It is best dissolved in oil of turpentine, and is used for mounting blood smears stained with Wright's blood stain. Colophonium mounting medium is prepared in the same manner as gum dammar.

Kaiser's glycerin jelly is very useful to preserve sections stained with scharlach R for fat, and with methyl violet for amyloid.

Gelatin (French sheet) -----	10 Gm.
Distilled water -----	60 c.c.
Pure glycerin -----	70 c.c.
Carbolic acid -----	1 c.c.

Heat the water in a beaker over a free flame or in a water bath, and dissolve the gelatin. When gelatin is dissolved, add the glycerin and carbolic acid, and stir the mixture until smooth. Then filter through wet absorbent cotton while hot, and store it in a cork-stoppered bottle. To melt it, place the bottle in the paraffin oven for 15 minutes.

Gum Arabic

Pure gum arabic -----	50	Gm.
Pure cane sugar -----	50	Gm.
Distilled water -----	50	c.c.
Thymol crystals -----	0.5	Gm.

Dissolve gum arabic and sugar separately and mix together, then add thymol crystals to the mixture. Keep in well-stoppered bottle. It sets quite hard.

Temporary Mounting Media.—For temporary mounts, cedar oil or glycerin is used, and the slides are discarded after emergency examination.

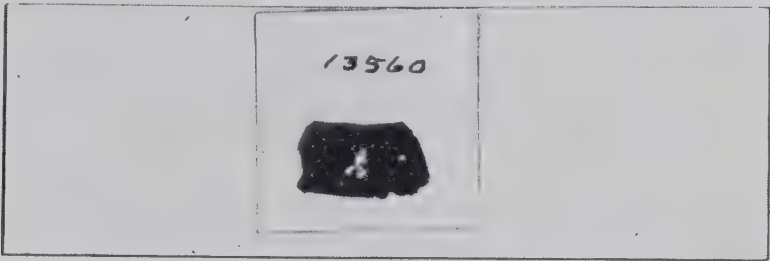


Fig. 82.—Identification number under cover slip.

Identification Number Under Cover Slip

A very convenient method of labeling slides is shown in Fig. 82. Print in small letters the laboratory specimen number or the name of the patient on very fine tissue paper with India ink (Higgins), and cut out a square with scissors. Put a drop of gum dammar over the section, place the printed number alongside it, and cover the slide with a cover slip.

PART VII

STAINING METHODS

THEORY AND HISTORY OF STAINING

The theory of staining is complex, and no satisfactory explanation has been advanced for the various effects, except that they depend on factors of chemical and physical nature. Because the physical chemistry of the cell and tissue elements is not well understood, it is not clear why certain tissues have an affinity for one dye and not for the other.

History.—The use of dyes in histological microscopy began about 1850. Carmine, indigo, iodine, fuchsin, aniline red, aniline violet, and other dyes were used in simple solutions. Hematoxylin was first introduced by Waldeyer to stain axis cylinders in an aqueous solution. In 1865 Bohmer combined hematoxylin with alum, and thereby made it a valuable histological stain. This opened the way for more combination stains. Later, about 1890, the differential stains were introduced with the use of various chemical reagents.

Progress continued, and every year new staining methods of considerable importance were introduced.

Mallory developed his famous aniline blue stain for connective tissue; Heidenhain, his iron hematoxylin for protozoological work; Levaditi discovered a stain for *Spirochaeta pallida* in tissue; Cajal revolutionized the subject of neuropathology by his investigations and discovery of various staining methods for nerve fibers.

Other investigators and authors have developed important methods for the identification of such elements in tissue as glycogen, amyloid, fat, and many others.

STAINING METHODS

The aim of staining is to recognize different tissue elements by their color reactions.

Staining methods may be classified under three groups: 1. Vital staining. 2. Routine staining method. 3. Special or differential staining methods.

A routine method is one which stains various types of protoplasm generally, with little differentiation except between nucleus and cytoplasm. It demonstrates the cells and the general cellular arrangement of the tissue.

Special staining methods are those of more limited scope, which demonstrate or differentiate special features of the tissue, such as the minute intra- or intercellular structure, foreign materials, organisms, etc.

Vital Staining

Vital staining is employed to stain living cells, injecting the staining solution into any part of the animal body. It causes a specific coloration of the cytoplasm of certain cells, chiefly those of the reticulo-endothelial system.

In employing vital stains, a sterile syringe and needle are used. The staining solution to be injected should be free from particles or clumps, the presence of which might cause emboli in the capillary vessels.

The vital dyes are usually injected intravenously, intraperitoneally, or subcutaneously. Smaller doses are needed for intravenous administrations, but large doses are necessary for subcutaneous and intraperitoneal injections.

Technic of Vital Staining

Lithium Carmine.—A concentrated suspension of carmine (about 5 Gm. per 100 c.c.) in cold saturated lithium carbonate is filtered and sterilized, and 5 to 10 c.c. injected intravenously in rabbits.

India Ink (Higgins).—Dilute the India ink with an equal volume of sterile distilled water in a sterile test tube and warm it to the body temperature before injecting. The average intravenous dose is about 5 c.c. for rabbits, repeated daily for 3 to 4 days, and then every three days thereafter.

Supravital Stains

Supravital stains are used to stain the living cell immediately after it is removed from the (living) body.

Thin slices of tissue are placed in small staining dishes, and enough staining solution is added to cover the tissue.

Several dyes are in general use.

Neutral red, a basic dye, is useful for the observation of the cell granules and vacuoles of phagocytic cells. It is employed on slices

of tissue in the strength of 1:10,000 to 1:100,000. Another method is to kill the animal by bleeding and inject a 1:15,000 solution of dye in physiological salt solution.

Janus green is regarded as a specific stain for mitochondria.

Stain thin slices of tissue in Janus green B in physiological salt solution 1:10,000. Specific mitochondria stain may be given with a weaker solution, 1:500,000 dilution.

Janus green B, in the strength of 1:10,000 in physiological salt solution, can be injected in animals previously bled to death.

Trypan Blue.—For use, 1 Gm. of dye is suspended in 100 c.c. of sterile distilled water and used immediately. It is dangerous to allow the suspension to stand more than an hour because it is likely to become toxic.

Isamine blue and *Niagara blue* give similar results and are used in the same manner as trypan blue.

Combination of Vital Stains.—It is possible to combine two or more types of vital stains, if so desired. One may inject India ink intravenously, trypan blue intraperitoneally, and lithium carmine intrapleurally.

Some cells will ingest one dye, others may take up all dyes.

ROUTINE NUCLEAR STAIN

Staining Method for Frozen Sections

1. Frozen sections of thoroughly fixed tissues, after being attached to the slide by a thin film of celloidin (described elsewhere), are stained in one of the alum hematoxylin stains (Delafield's or Harris') for from 2 to 5 minutes. Harris' alum hematoxylin is preferable.

2. Wash in a beaker containing tap water.

3. Destain in acid alcohol (1 c.c. hydrochloric acid in 99 c.c. 70 per cent alcohol) until reddish color of hematoxylin ceases to come off.

4. Wash in a large basin of tap water.

5. Blue the section by dipping in 2 per cent ammonia water (2 c.c. ammonium hydroxide in 98 c.c. tap water).

6. Wash in a large basin of water.

7. Stain sections in 1 per cent aqueous solution of eosin for 5 to 10 minutes.

8. Wash in 2 changes of tap water.
9. Dehydrate thoroughly in 2 changes of absolute alcohol or anhydrous isopropanol (dropping bottle method), 2 minutes each.
10. Place in 2 changes of carbolxylol, 3 minutes each.
11. Clear in pure xylol for 5 minutes.
12. Clear in second pure xylol for 5 minutes.
13. Mount in gum dammar.

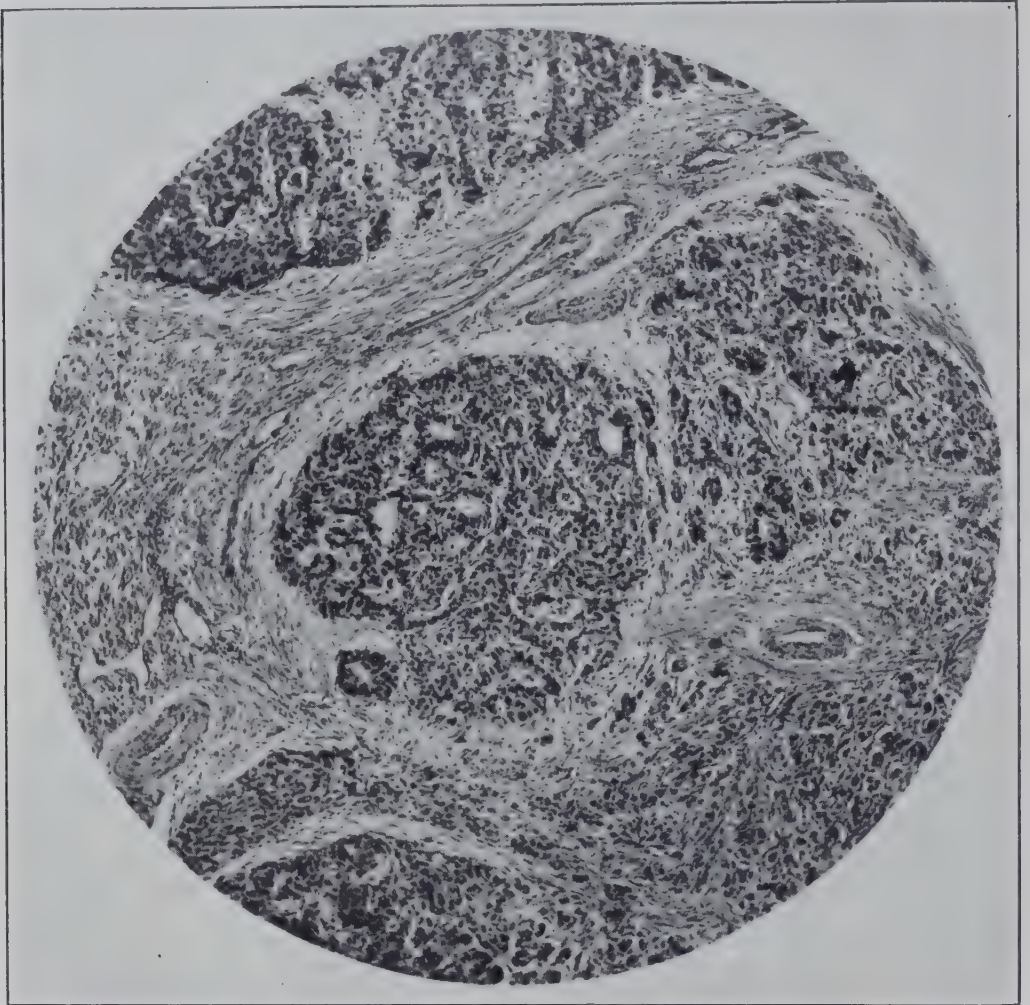


Fig. 83.—Cirrhosis of liver. Hematoxylin-eosin stain. Frozen section.

Eosinol

(Staining Method)

1. Prepare microscopic sections by one of the standard sectioning methods (i.e., employing frozen tissue or tissue embedded in paraffin or pyroxylin) and stain in alum hematoxylin (Harris or Delafield) for from 3 to 7 minutes.
2. Wash in tap water until blue (about 30 seconds).

3. Destain in acid alcohol (1 c.c. concentrated hydrochloric acid in 99 c.c. 70 per cent alcohol) by dipping the section in and out for even destaining.
4. Rinse in tap water.
5. Blue by dipping in 2 per cent ammonia water (2 c.c. strong ammonium hydroxide in 98 c.c. tap water).
6. Wash in tap water for a few seconds.

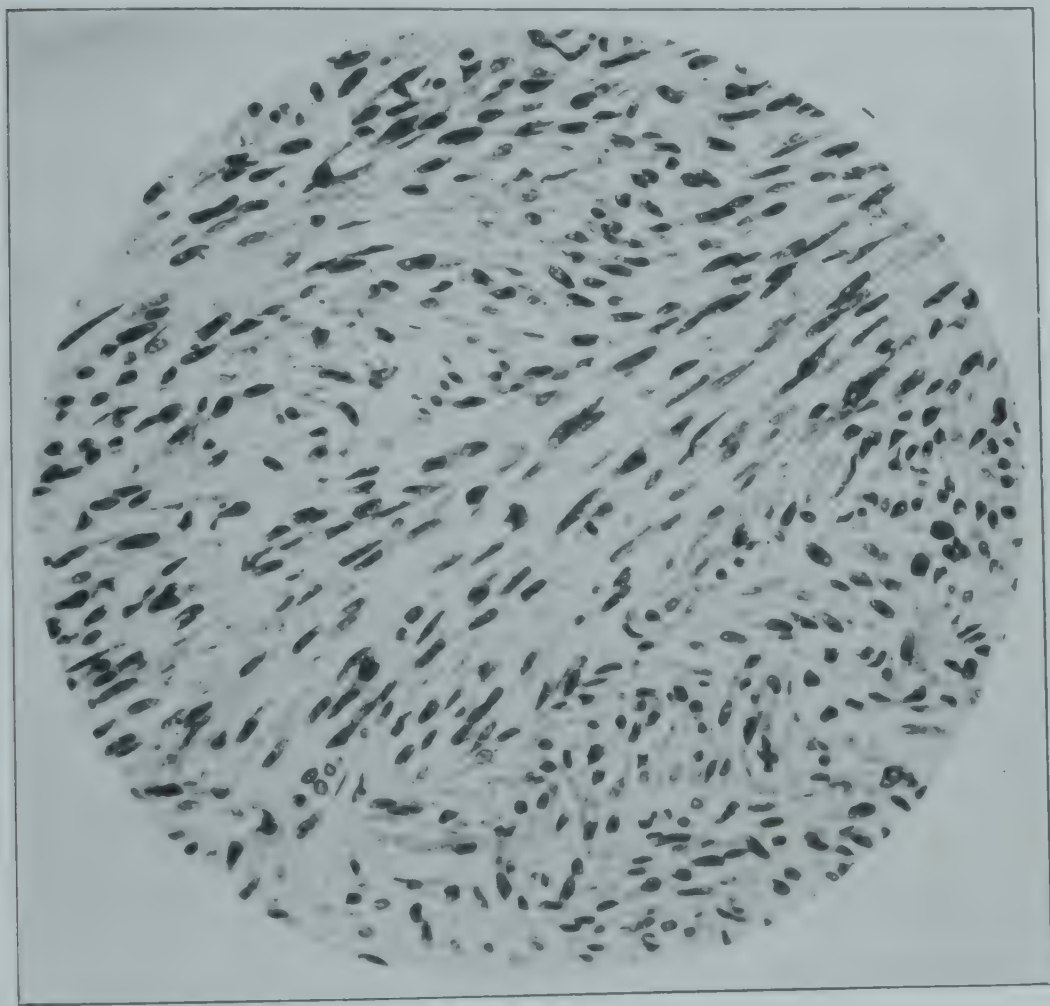


Fig. 84.—Fibroma of uterus. Hematoxylin-eosin stain. Frozen section.

7. Wipe off the water with a towel, dehydrate in 95 per cent alcohol for 1 minute, and wipe off the back of the slide.
8. Dehydrate completely by pouring on a few drops of absolute alcohol or anhydrous isopropanol from a drop bottle. Repeat.
9. Counterstain and partially clear in eosinol for from 10 to 30 seconds, depending on the strength of the solution.
10. Treat with carbolxylol for 3 minutes.
11. Treat with first xylol 2 minutes.

12. Treat with second xylol 2 minutes.
13. Treat with third xylol 2 minutes.
14. Mount in gum dammar (saturated solution of gum dammar in neutral histologic xylol).

Method to Remove Wrinkles From Creased Sections

Sometimes sections of brain, liver, and spleen are creased when submitted to clearing in carbolxylol or in fresh xylol. To flatten them out, blot section thoroughly, dip in thin celloidin twice, place in carbolxylol for 1 minute, blot thoroughly in filter paper, clear in xylol in usual manner, and mount in gum dammar.

Staining Method for Paraffin Sections

Paraffin sections are always stained on the slide after they are attached and dried. Before staining, certain preparatory treatment is necessary:

Removing of Paraffin.—

1. Flood the slide with pure xylol from a dropping bottle, placing sections on staining dishes for 5 minutes. Repeat the process.

2. Pour off the second xylol, and flood the slide with absolute alcohol or anhydrous isopropanol twice, 5 minutes each.

3. If the tissue is fixed in Zenker's fluid or some other fluid containing bichloride of mercury, the sections should be treated first in tincture of iodine for 5 minutes in order to remove the mercury precipitate, followed by a solution of sodium thiosulfate (hypo), until the natural color of the section reappears.

Sodium thiosulfate	0.75 Gm.
Alcohol, 95 per cent	10 c.c.
Water	90 c.c.

4. Wash in tap water.

5. Stain in Delafield's or Harris' alum hematoxylin (Harris' preferable) for from 7 to 10 minutes.

6. Wash in a beaker of water until blue.

7. Destain in acid alcohol (1 c.c. hydrochloric acid in 99 c.c. 70 per cent alcohol) until reddish color of hematoxylin ceases to come off.

8. Wash in a large basin of tap water.

9. Blue the sections by dipping in 2 per cent ammonia water (2 c.c. ammonium hydroxide in 98 c.c. tap water).

10. Rinse in tap water.

11. Stain in 1 per cent aqueous solution of eosin for from 5 to 10 minutes, or follow eosinol technic.
12. Wash in 2 changes of tap water.
13. Dehydrate thoroughly in 2 changes of absolute or butyl alcohol or anhydrous isopropanol, 2 minutes each.
14. Place in 2 changes of carbolxylol, 3 minutes each.
15. Clear in pure xylol for 5 minutes.
16. Clear in a second pure xylol 5 minutes or longer.
17. Mount in gum dammar.

Eosin-Methylene Blue Stain

Zenker's and mercuric chloride fixed tissue sections give the best results. The sections are stained in the following manner.

1. Deparaffinize sections by 2 applications of xylol, 2 minutes each.
2. Remove xylol with 2 applications of absolute alcohol, 2 minutes each.
3. Wash thoroughly in tap water.
4. Place the section in a jar containing 5 per cent eosin Y, and leave it in paraffin oven for 30 minutes at 52° C.
5. Rinse rapidly in tap water, and immediately place in Borrel's methylene blue; then leave it in paraffin oven for 30 minutes at 52° C.
6. Wash in tap water. The section should be blue-black.
7. Differentiate in colophonium-alcohol (0.5 c.c. colophonium in 100 c.c. alcohol), agitating the slide constantly and examining every few seconds under microscope.
8. Dehydrate rapidly in absolute alcohol.
9. Clear in xylol 5 minutes.
10. Mount in gum dammar.

The result: cell nuclei, blue; cytoplasm, collagen fibers, and muscle, pink; erythrocytes, bright pink, likewise eosinophiles.

The formula for Borrel's methylene blue solution is as follows:

1. Prepare 1 per cent solution of methylene blue in distilled water and heat in water bath.
2. Dissolve 0.5 Gm. of silver nitrate in 100 c.c. of distilled water, placing the solution in 500 c.c. capacity flask.
3. Prepare a 3 per cent solution of sodium hydrate, and slowly add to the silver solution, shaking the flask constantly. A brown precipitate of silver oxide is formed. Stop adding the sodium hydrate solution as soon as no more precipitate comes down. Let the

precipitate settle, discard the supernatant fluid, and wash the precipitate by shaking in distilled water. Repeat this several times until the distilled water is no longer alkaline to litmus paper. Pour off the distilled water and add the 1 per cent methylene blue solution. Boil this mixture gently for 5 minutes and cool. The solution will assume a dark violet color when cold. If still blue, boil it further to oxidize methylene blue. If it remains reddish, add a little more methylene blue, until violet. Filter the solution and store in stoppered bottle. Dilute the above 1 part stain to 5 parts distilled water at the time of use.

Staining Method for Celloidin Sections

The following is the staining method for celloidin sections:

1. Stain loose sections in a Stender dish containing Harris' hematoxylin, 2 to 5 minutes.
2. Wash in tap water until blue.
3. Destain in acid alcohol until reddish color of hematoxylin ceases to come off.
4. Dip in ammonia water until blue.
5. Stain in 1 per cent aqueous eosin for from 5 to 10 minutes, or in eosinol for from 10 to 20 seconds.
6. Wash in 2 changes of tap water.
7. Place in graded alcohols, 70 per cent, 80 per cent, and 90 per cent for few minutes.
8. Place in 90 per cent alcohol for few minutes.
9. Place sections in the following solution for not more than 5 minutes.

Xylol	-----	1 part
Chloroform	-----	1 part
Absolute alcohol	-----	1 part

10. Transfer the sections to a dish of pure beechwood creosote. If wrinkled, they will immediately flatten out. Do not leave in the solution more than 10 minutes, as celloidin is slightly soluble in creosote and alcohol.

11. Transfer the sections on glass slides.
12. Blot in filter paper.
13. Pour pure xylol on the slide, drain it off and repeat the process once or twice. Do this as rapidly as you can, as prolonged treatment with xylol will cause sections to crease.
14. Blot rapidly with filter paper.
15. Mount in gum dammar or Perm'ount.

PART VIII

SPECIAL OR DIFFERENTIAL STAINING METHODS

Collagen Fibrils and Reticulum of Connective Tissue Mallory's Aniline Blue Stain (Modified)

Mallory's aniline blue stain is not absolutely differential, because in addition to collagen fibrils and reticulum, it also stains hyaline, fibrin, fibroglia fibrils, smooth and striated muscle fibers, and amyloid. The method:

1. Fix tissues in Zenker's fluid.
2. Embed in celloidin or paraffin.
3. Cut thin sections. Flood slides with xylol for 5 minutes. Repeat. Then flood slides with absolute alcohol or anhydrous isopropanol for 3 minutes. Repeat. Rinse in water and place in tincture of iodine for 5 minutes. Rinse in alcohol, then water. Bleach with 5 per cent sodium thiosulfate for 5 minutes. Rinse thoroughly in water.
4. Place in 1 per cent solution of acid fuchsin for from 5 to 10 minutes.
5. Rinse quickly in tap water.
6. Place in 1 per cent aqueous solution of phosphomolybdic acid for 1 minute.
7. Rinse in water.
8. Stain for 5 minutes in the following solution:

Aniline blue (water soluble) -----	0.5 Gm.
Orange G -----	2 Gm.
Oxalic acid -----	2 Gm.
Water -----	100 c.c.

9. Rinse in tap water and blot.
10. Dehydrate thoroughly with absolute alcohol or anhydrous isopropanol.
11. Clear in xylol for 5 minutes. Repeat in fresh xylol.
12. Mount in gum dammar.

For celloidin sections, use 95 per cent alcohol and clear in xylol by blotting method.

The collagen fibrils, reticulum, connective tissue, amyloid, mucus, and hyaline stain blue; nuclei, cytoplasm, fibroglia fibrils, axis cylinders, neuroglia fibers, and fibrin, red; red blood corpuscles and myelin sheaths, yellow; elastic fibers, pale pink or yellow.

If it is desired to bring out the collagen fibrils as sharply as possible, omit the staining with acid fuchsin. Then the nuclei and protoplasm stain yellow, and the blue fibrilla and reticulum stand out more prominently.

This is an excellent and colorful method to demonstrate connective tissue fibers.

Azocarmine Stain

(Heidenhain's Modification of Mallory's Aniline Blue)

1. Fix tissue in Zenker's solution for 16 hours. Wash overnight. Run it up through graded alcohols:

80 per cent alcohol, overnight
95 per cent alcohol, the next day
Absolute alcohol, overnight

2. Xylol for 2 or more hours until tissue is clear. Place in oven at 56° C. for one-half hour. Then change paraffin. Leave in oven for 3 to 4 hours. Embed. Cut and mount sections and dry in oven at 45° C. for from 3 to 7 days. Run slides down as follows:

Xylol	5 minutes
Absolute alcohol	3 minutes
95 per cent alcohol	3 minutes
80 per cent alcohol	3 minutes
Distilled water	3 minutes

Note.—Deformalinization, if necessary, should be done at this stage, as follows:

Place sections in ammonia water (40 drops of ammonia to 100 c.c. of distilled water). Leave in this solution for 7 hours.

Wash in tap water	1 hour
Helly's or Zenker's fluid	overnight
Wash in tap water	1 hour

This step is used only if tissues have been fixed in formalin.

3. Take crystals out with Lugol's solution for 1 minute, and then sodium hyposulfite (2 per cent) for 2 minutes. Wash in tap water for 5 minutes.

4. Stain sections in azocarmine in oven at 56° C. for from 45 to 60 minutes. Allow to cool 5 to 10 minutes at room temperature.

Preparation of the stain:

Azocarmine	1 Gm.
Distilled water	100 c.c.
Heat 20 minutes, cool and filter	
Acidify with 1 c.c. glacial acetic acid	

5. Wash in distilled water.

6. Differentiate in aniline alcohol, 1 to 3 minutes.

Aniline oil	1 c.c.
95 per cent alcohol	100 c.c.

Nuclei stain red and cytoplasm stains pale pink (control under microscope).

7. Remove aniline with alcohol, acidified with 1 per cent acetic acid, for one-half minute. (If section is too red, put back in aniline-alcohol solution for a few seconds.)

8. Phosphotungstic acid, 5 per cent, for from 2 to 3 hours.

9. Wash quickly in distilled water.

10. Mallory's aniline blue-orange G-acetic acid solution for from 3 to 6 hours.

Aniline blue (water soluble)	0.5 Gm.
Acetic acid, glacial	8 c.c.
Orange G	2 Gm.
Distilled water	100 c.c.

Heat and filter the mixture after cooling, and thin with as much water as there is stain.

11. Wash in distilled water.

12. Differentiate in absolute alcohol, controlling under the microscope.

13. Clear in xylol.

14. Mount in gum dammar.

Amyloid, connective tissue, and mucous colloid take deep blue; nuclei, red.

This is a very valuable stain to show minute details of connective tissue.

A New Aniline Blue Stain for Connective Tissue and Hyaline, Applicable to Frozen and Paraffin Sections of Formaldehyde-Fixed Tissue

(Krajian)

Method for Frozen Sections

1. Fix tissues in 10 per cent neutral formaldehyde in the usual manner. For emergency examination, fix in hot formaldehyde in paraffin oven (56° C.) for 15 minutes.

2. Cut thin frozen sections.

3. Place in the following solution (mordant) for 5 minutes:

Saturated aqueous solution of picric acid -----	25 c.c.
Alcohol, 95 per cent -----	25 c.c.
Acetone -----	50 c.c.
Thorium nitrate -----	4 Gm.
Cadmium nitrate -----	5 Gm.

Dissolve thorium nitrate and cadmium nitrate in picric acid solution first, then add alcohol and acetone.

4. Rinse in tap water and transfer section to a glass slide.

5. Dehydrate with anhydrous isopropanol or absolute alcohol for 15 seconds.

6. Blot and dip once in thin celloidin.

7. Dip in tap water and apply the following staining solution for 5 minutes:

Azocarmine -----	1 Gm.
Distilled water -----	100 c.c.

Heat 10 minutes, cool and filter. Acidify with 1 c.c. glacial acetic acid.

Take 1 part of the above staining solution and mix with an equal volume of 2 per cent aqueous solution of acid fuchsin.

8. Rinse rapidly in tap water and treat with a 10 per cent aqueous solution of phosphotungstic acid for 3 minutes.

9. Rinse in tap water and stain in the following aniline blue solution for from 5 to 8 minutes:

Aniline blue (water soluble) -----	0.5 Gm.
Orange G -----	2 Gm.
Acetic acid, glacial -----	8 c.c.
Distilled water -----	100 c.c.

Take 2 volumes of the aniline blue solution and mix with 1 part of 10 per cent aqueous citric acid.

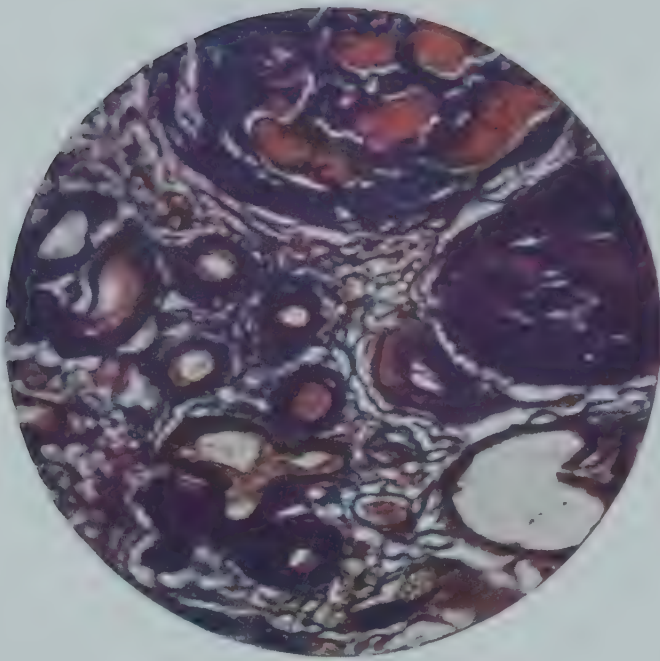
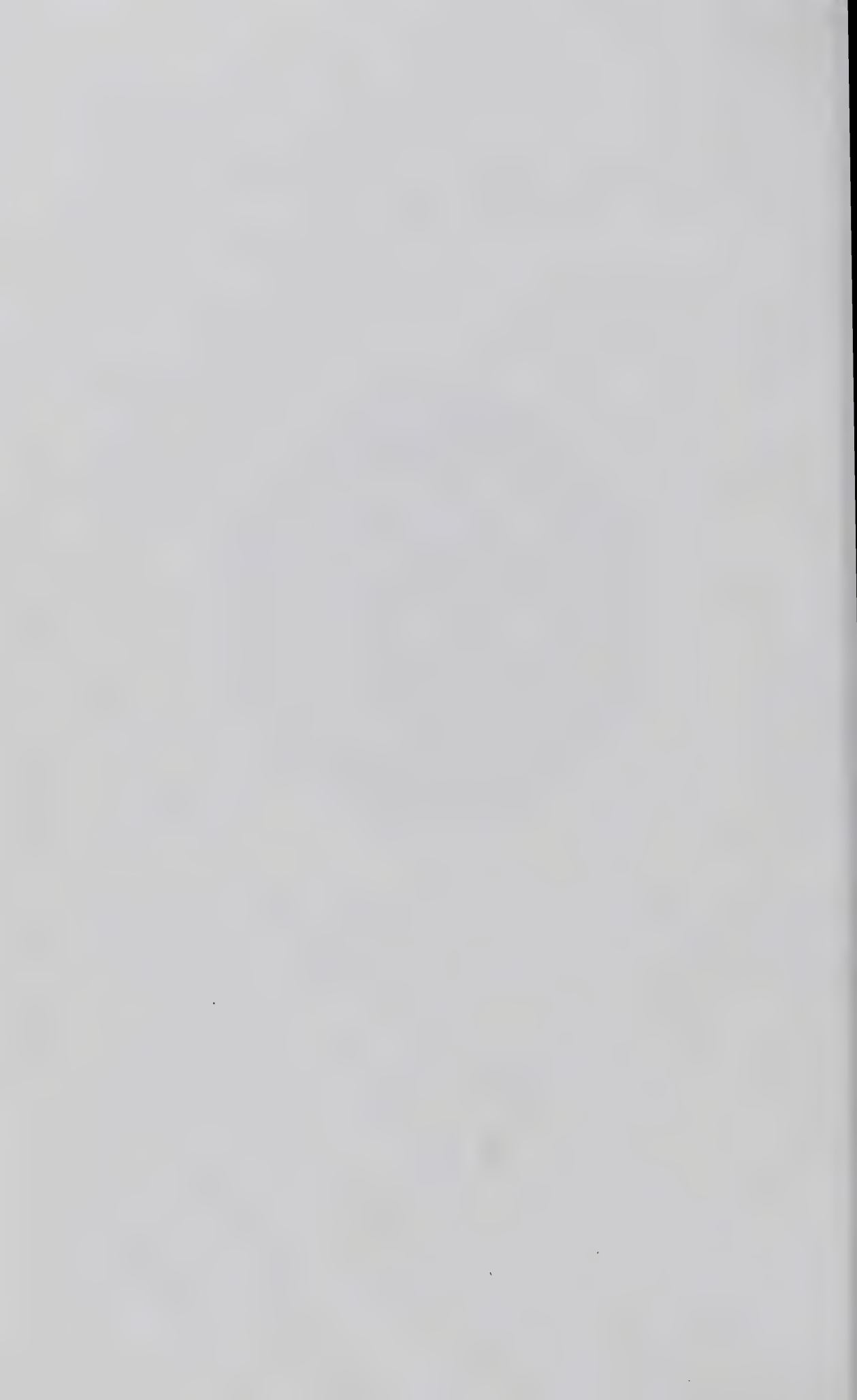


PLATE I

Modified aniline blue stain on a kidney section from a case of glomerulonephritis. Collagen fibers, dark blue; reticulum, light blue; erythrocytes, golden yellow; nuclei, red; elastic fibers, dark red. Paraffin section.

Magnification 250 diameters. Original on Kodachrome. Made with Bausch & Lomb 8.3 MM apochromatic objective of 0.65 N.A. and 7.5X compensating eyepiece.



10. Wash rapidly in tap water and control under microscope. If deeper shade of blue is desired, continue treatment with aniline blue until desired result is obtained.

11. Dehydrate rapidly with 3 applications of anhydrous isopropanol or absolute alcohol and follow with butyl alcohol for 2 minutes.

12. Clear in 2 changes of xylol, 1 minute each.

13. Mount in gum dammar.

Collagen fibers stain deep blue; reticulum, light blue; hyaline, bright red; nuclei, red; erythrocytes, golden yellow; elastic fibers, dark red.

All the solutions should be kept in tightly stoppered bottles. They can all be used at least a dozen times, except aniline blue-citric acid combination, which should be discarded after it is used.

Method for Paraffin Sections

1. Fix tissues in 10 per cent neutral formaldehyde in the usual manner.

2. Cut thin paraffin sections.

3. Deparaffinize with 2 applications of xylol, 2 minutes each, follow with 3 applications of anhydrous isopropanol or absolute ethyl alcohol.

4. Wash in tap water and place in the following solution (mordant) for 10 minutes:

Saturated aqueous solution of pierie acid	25 c.c.
Alcohol, 95 per cent	25 c.c.
Acetone	50 c.c.
Thorium nitrate	4 Gm.
Cadmium nitrate	5 Gm.

Dissolve thorium nitrate and cadmium nitrate in pierie acid solution first, then add alcohol and acetone.

5. Rinse in tap water and apply the following staining solution for 10 minutes:

Azocarmine	1 Gm.
Distilled water	100 c.c.

Heat 10 minutes, cool and filter. Acidify with 1 c.c. glacial acetic acid.

Take 1 part of the above staining solution and mix with an equal volume of a 2 per cent aqueous solution of acid fuchsin.

6. Rinse in tap water and treat with a 10 per cent aqueous solution of phosphotungstic acid for 5 minutes.

7. Rinse in tap water and stain in the following aniline blue solution for from 8 to 12 minutes:

Aniline blue (water soluble) -----	0.5 Gm.
Acetic acid, glacial -----	8 c.c.
Orange G -----	2 Gm.
Distilled water -----	100 c.c.

Take 2 volumes of aniline blue solution and mix with 1 part of 10 per cent citric acid.

8. Wash rapidly in tap water and control under microscope. If deeper shade of blue is desired, continue staining with aniline blue until desired result is obtained.

9. Dehydrate rapidly with 3 applications of anhydrous isopropanol or absolute alcohol and follow with butyl alcohol for 2 minutes.

10. Clear in 2 changes of xylol, 1 minute each.

11. Mount in gum dammar.

Van Gieson Stain

The van Gieson stain is a selective method to demonstrate collagen fibers of connective tissue.

1. Stain frozen, paraffin, or celloidin sections rather deeply with alum hematoxylin (Harris).

2. Wash in water until blue.

3. Stain in van Gieson's solution for 5 minutes.

4. Transfer directly to 95 per cent alcohol followed by absolute alcohol.

5. Clear in xylol and mount in gum dammar or balsam. For celloidin sections, clear in oil origanum after 95 per cent alcohol.

By this method, cell nuclei stain dark brown; collagen fibers, bright red; muscle, red corpuscles, and other tissues, a transparent yellow.

Krajan Modification of Van Gieson Stain

1. Make frozen, paraffin, or celloidin sections.

2. Stain 5 minutes in the following elastic tissue solution:

Hematoxylin crystals -----	3 Gm.
Alcohol, 95 per cent -----	25 c.c.
Potassium iodide -----	3 Gm.
Ferric chloride -----	3 Gm.
Ferric ammonium sulfate -----	3 Gm.
Distilled water -----	25 c.c.

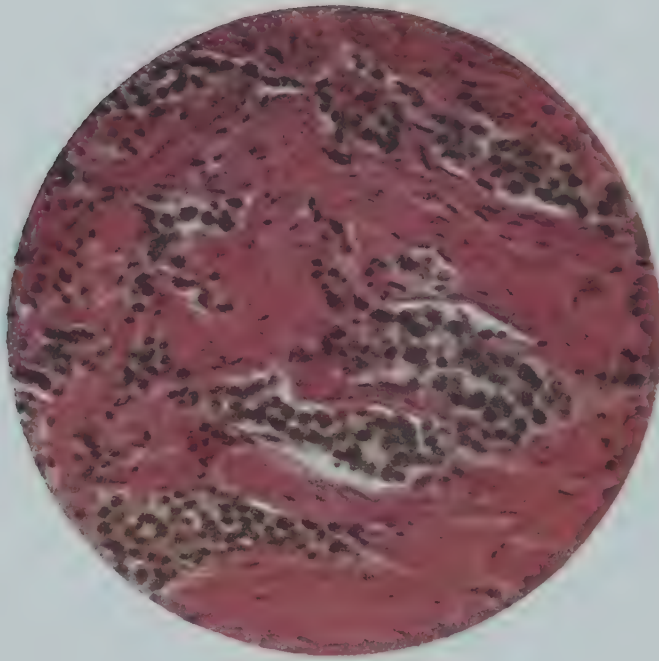


PLATE II

Modified van Gieson stain on a uterus section from a case of carcinoma of uterus. Cancer cells, dark brown; connective tissue, red. Frozen section.

Magnification 250 diameters. Original on Kodachrome. Made with Bausch & Lomb 8.3 MM apochromatic objective of 0.65 N.A. and 7.5X compensating eyepiece.

Dissolve hematoxylin crystals in alcohol. Dissolve other reagents in water and mix with hematoxylin solution.

3. Wash in tap water.
4. Destain in acid alcohol (1 c.c. concentrated hydrochloric acid in 99 c.c. 70 per cent alcohol) until no more color comes out.
5. Rinse in water.
6. Stain in van Gieson solution for 5 to 10 minutes.
7. Rinse rapidly in water.
8. Blot dry.
9. Differentiate with oil origanum-alcohol (equal parts of oil origanum and absolute alcohol or isopropanol) for 5 minutes, changing the solution twice.
10. Clear in xylol and mount in gum dammar.

The elastic tissue staining solution keeps well for long periods in well-stoppered bottles. It acts as a mordant in van Gieson stain; it stains elastic tissue fibers, myelin sheath, and bacteria.

Trichrome Stain of Cajal for Connective Tissue

(Modification of Ramon Castreviejo)

1. Fix tissue in 10 per cent formaldehyde.
2. Prepare frozen, paraffin, or celloidin sections.
3. Stain in acetic fuchsin formalin for 2 to 5 minutes.

Ziehl-Neelsen carbolfuchsin	15 drops
Glacial acetic acid	2 drops
Formaldehyde, 40 per cent	2 drops

4. Wash in water.
5. Stain in following solution for 5 to 10 minutes:

Indigo carmine	0.25 Gm.
Saturated aqueous solution of picric acid	100 c.c.
Glacial acetic acid	5 c.c.

6. Wash rapidly in water.
7. Dehydrate in 95 per cent alcohol, follow with absolute alcohol.
8. Clear in carbolxylol, follow with xylol.
9. Mount in gum dammar.

Nuclei stain deep red violet; cartilage, mucin, and mast cells, very intense bluish violet; cytoplasm, clear green or yellowish green; connective tissue, intense blue; and muscle fibers, clear green. A valuable connective tissue stain.

Perdrau-Bielschowsky Method for Reticulum

1. Fix tissue in 10 per cent formaldehyde.
2. Cut frozen sections, 15 to 20 microns thick.
3. Leave in distilled water 24 hours.
4. Treat sections for 10 minutes in 0.25 per cent potassium permanganate.
5. Wash and place in:

Oxalic acid -----	1 Gm.
Potassium sulfite -----	1 Gm.
Distilled water -----	200 c.c.

Decolorize until white.

6. Wash thoroughly in several changes of distilled water overnight.
7. Place in 2 per cent silver nitrate solution in the dark for 1 to 2 days.
8. Wash in distilled water for not more than 5 minutes.
9. Treat sections 40 to 60 minutes in Bielschowsky's ammonium silver solution:

Silver nitrate, 10 per cent -----	5 c.c.
Sodium hydroxide, 40 per cent -----	5 drops
Distilled water -----	40 c.c.

Add strong ammonia drop by drop until precipitate is almost completely dissolved.

10. Wash quickly in distilled water.
11. Reduce in 20 per cent formaldehyde for 30 minutes.
12. Wash in distilled water. Tone in 0.2 per cent yellow gold chloride solution.
13. Wash in distilled water. Fix in sodium hyposulfite.
14. Wash, dehydrate, clear, and mount.

This is an excellent but slow method of demonstrating reticulum fibers.

A Rapid and Dependable Method for Staining Reticulum Fibers in Frozen Sections

(Krajian)

The method:

1. Cut frozen sections, 7 to 10 microns.
2. Rinse in distilled water.

3. Place in the following solution for 5 minutes:

Potassium permanganate, 0.3 per cent aqueous solution	15 c.c.
Strong ammonium hydroxide --	5 c.c.

4. Rinse in distilled water for a few seconds.

5. Decolorize in 2 per cent oxalic acid until all the brown color has disappeared (sections should not be left in this solution longer than necessary). Each section should be treated separately.

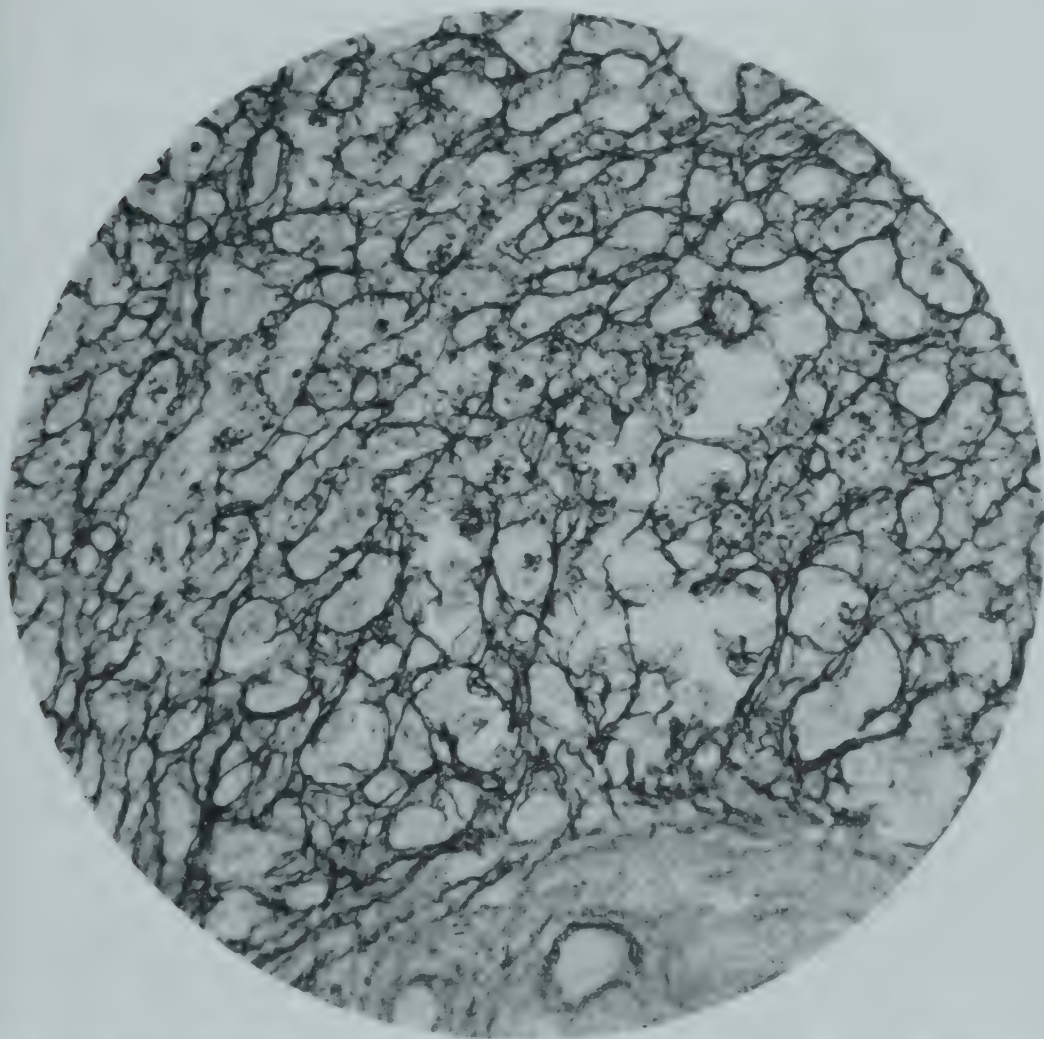


Fig. 85.—Liver section stained by Krajan's rapid reticulum stain. Fibers of reticulum stain jet black. Frozen section.

6. Wash in 2 changes of distilled water.

7. Place in warm ammoniacal silver solution, previously heated to 60° C. for 15 minutes (in paraffin oven).

The ammoniacal silver solution: Take a 10 per cent silver nitrate solution (about 20 c.c.) and add strong ammonium hydroxide drop

by drop until precipitate that is formed is almost completely dissolved.

8. Wash in 2 changes of distilled water.

9. Reduce in a warm 20 per cent solution of formaldehyde for 2 minutes (heated to 55° C.).

10. Rinse in distilled water.

11. Treat in 1:300 gold chloride solution for 2 minutes.

12. Rinse in distilled water.

13. Treat with 5 per cent sodium thiosulfate solution for 2 minutes.

14. Wash and transfer to glass slide.

15. When well drained off, mount in warm glycerin jelly.

A rapid and colorful method.

Foot Method for Staining Reticulum

(A rapid method for staining paraffin sections)

1. Fix tissues in Zenker's for 24 hours.

2. Wash under running water, 12 to 24 hours.

3. Dehydrate and embed in paraffin in the usual manner.

4. Cut thin sections, 5 to 7 microns thick, and dry them thoroughly in the paraffin oven 12 hours or longer.

5. Dissolve paraffin, using 2 changes of xylol. Wash in alcohol.

6. Tincture of iodine, 5 minutes.

7. Wash in alcohol and bleach in 5 per cent sodium thiosulfate.

8. Potassium permanganate, 0.25 per cent solution for 5 minutes.

9. Aqueous 5 per cent oxalic acid for 10 minutes, wash in tap water and then in distilled water.

10. Treat with silver ammonium carbonate solution, made fresh each time. To 10 c.c. of a 10 per cent aqueous solution of silver nitrate, add 10 c.c. of a saturated solution of lithium carbonate (use distilled water for both).

Allow the resulting heavy precipitate of silver carbonate to settle, and pour off the supernatant fluid. The precipitate is then washed several times in from 25 to 50 c.c. distilled water, and allowed to settle each time before the supernatant fluid is decanted. After 25 c.c. of fresh distilled water are added, the washed precipitate is almost dissolved in strong ammonia water, which is added drop by drop while the container is shaken continuously. The

precipitate turns grayish brown as the ammonia is added, about 8 to 15 drops of ammonium hydroxide being needed, depending on its concentration. The entire solution is then made up to 100 c.c. with distilled water, and heated to about 50° C. The slides are immersed in this solution and placed in the incubator at 37° C. for 10 to 15 minutes.

11. Wash quickly in distilled water and place the sections in a 10 per cent neutral formaldehyde solution for 2 minutes, until they become dark brown.

12. Wash in tap water and transfer sections in 1:500 gold chloride solution for 2 minutes.

13. Wash in water and fix in 5 per cent sodium thiosulfate for a few minutes.

14. Wash in water. If desired, a counterstain could also be given; if not, dehydrate the sections, clear in xylol, and mount in gum ammar.

A rapid and very useful method to demonstrate reticulum in paraffin sections.

Tumor Reticulum*

It is well known that the cells of the body are bound together by stroma or network of connective tissue fibers of which three varieties are universally recognized; namely, elastin, collagen, and reticulin. Collagen and reticulin appear to be closely related physically and chemically. Evidence is accumulating which may indicate that reticulin is a distinct substance formed by a precipitation-enzyme reaction in the secretions of certain cells such as reticulo-endothelial cells, histiocytes, monocytes, lymphocytes, fibroblasts, smooth and striated muscle cells, and vascular endothelium. Reticulin is apparently a precollagen since typical collagenous fibers may be formed by the impregnation of reticulin fibers with collagen.

Reticulum was first observed by Küpffer in 1876 during his histological investigation of the liver. It has been found that there is an increase in the sarcoma group of neoplasms rather than carcinomas. It is interesting to note, too, that the reticulum stain, as

*For much of the information regarding tumor reticulum, we are indebted to the book entitled, "Atlas of Tumor Reticulum" by R. P. Morhardt and A. A. Rajan. This brochure is published by Mac Printing Company, 2711 Raymond Ave., Los Angeles, Calif. Permission of authors and publisher has been granted to abstract this material.

developed by Morhardt and Krajian, impregnates epithelial cells whereas those neoplasms of connective tissue origin exhibit fibers only.

These authors are convinced that any undifferentiated cellular tumor which is without obvious characteristics is incompletely examined unless a reticulum stain is performed. They believe that carcinoma is never productive of an increase in reticulum, and that sarcomas provoke an increase in reticulum. Therefore, the use of reticulum stain offers a means of differentiation between sarcomas and carcinoma.

Morhardt and Krajian give a partial list of tumors, based on amount of reticulum found, as follows:

Increased Reticulum

Testicular seminoma
Pleural mesothelioma
Periphery of glioblastoma
Spinobasilar chordoma
Periphery of astrocytoma
Plasma-cell myeloma
Periphery of metastatic melanoblastoma in brain
Leiomyosarcoma
Leiomyoblastoma
Reticulum-cell sarcoma
Osteosarcoma
Lymphosarcoma
Retroperitoneal sarcoma, neurogenic
Giant-cell tumor, osteoclastoma
Neurofibrosarcoma
Testicular lymphosarcoma
Monocytic leucemia (liver)
Plasma-cell leucemia (liver)
Wilms's tumor (sarcomatous component)
Malignant lymphoma

Normal Reticulum

Carcinoma of lung, primary
Fibroadenoma of breast
Neuroma
Uterine adenomyosis
Hemangioma
Lipoma
Neurilemoma
Dysgerminoma (ovarian seminoma)

Carcinoma of thyroid gland
Adamantinoma
Carotid body tumor

Diminished Reticulum
(Displacement of normal tissue)

Hepatoma
Carcinoma of ovary
Carcinoma of testis
Neuroepithelioma
Glioblastoma, central portion
Glioma, central portion
Chorionepithelioma
Carcinoma, adrenal, metastatic
Medulloblastoma (center area)
Malignant melanoma, lymph node
Adrenal cortical adenoma
Ewing's tumor, lymph node
Carcinoma, pituitary, metastatic
Cystic disease of breast
Carcinoid in liver
Carcinoma of stomach

Staining Technic

A rapid and reliable method for the demonstration of reticulin fibers in frozen sections:

Fix blocks in 10 per cent formaldehyde.

1. Cut frozen section 7 to 10 microns.
2. Place in a warm 10 per cent solution of ammonium hydrate in paraffin oven at 56° C. for 10 minutes.
3. Wash rapidly in distilled water.
4. Place sections in 0.25 per cent solution of potassium permanganate for 10 minutes.
5. Wash in distilled water and decolorize in 3 per cent oxalic acid until most of the brown color is removed.
6. Wash in distilled water.
7. Place in a warm ammoniacal silver solution and put in paraffin oven for 40 minutes at 56° C.

Ammoniacal silver solution: 10 c.c. of 10 per cent silver nitrate in a Stender dish. Add strong ammonium hydrate drop by drop while agitating the container or while stirring with a glass rod until the precipitate disappears.

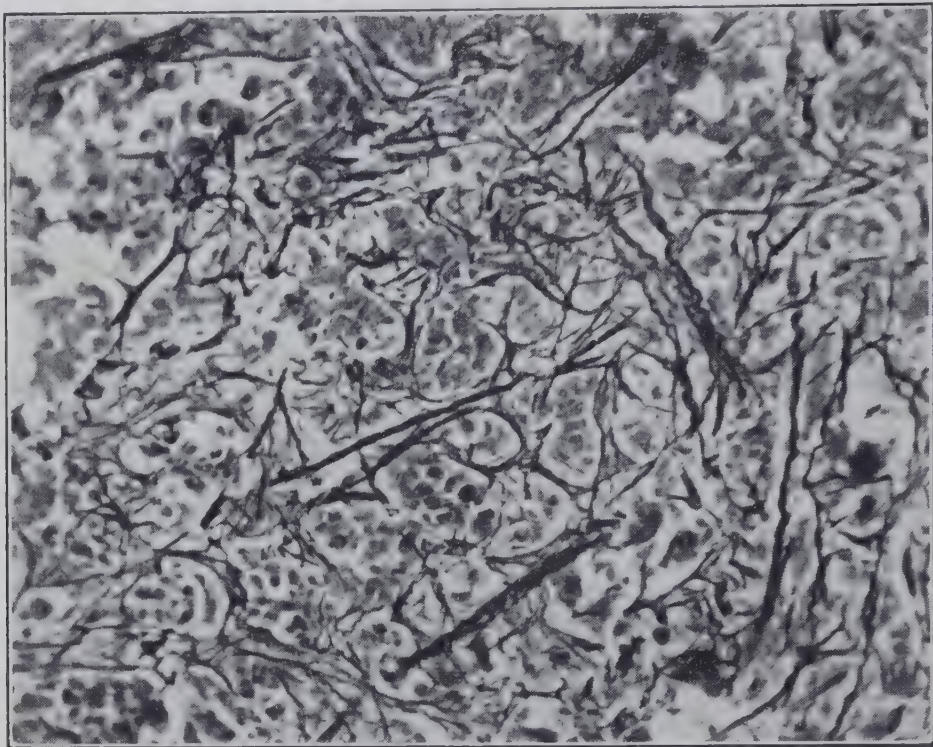


Fig. 86.—Carcinoma of stomach. Note the staining of carcinoma cells and absence of reticulum.

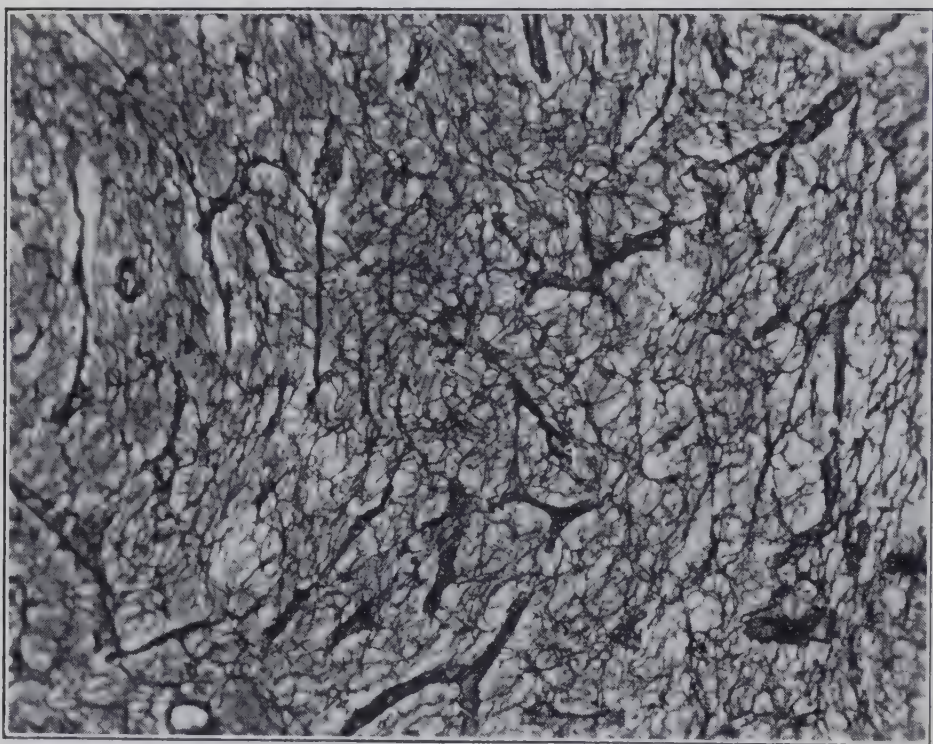


Fig. 87.—Lymphosarcoma, showing great abundance of reticulum and absence of cellular staining.

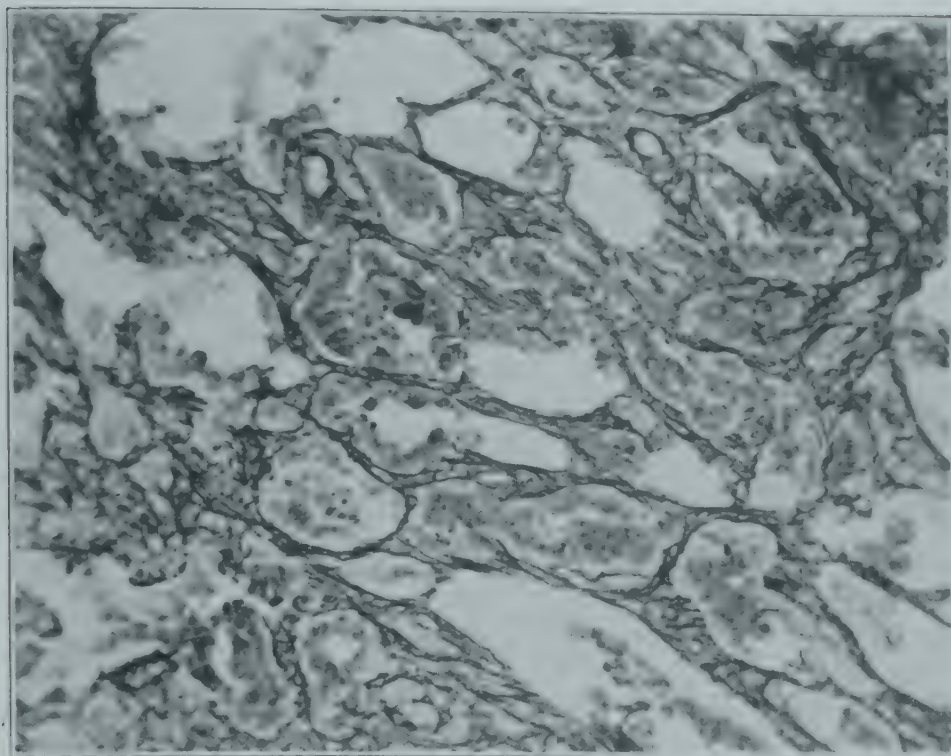


Fig. 88.—Carcinoma of lung. Note the staining of cells and absence of reticulum.

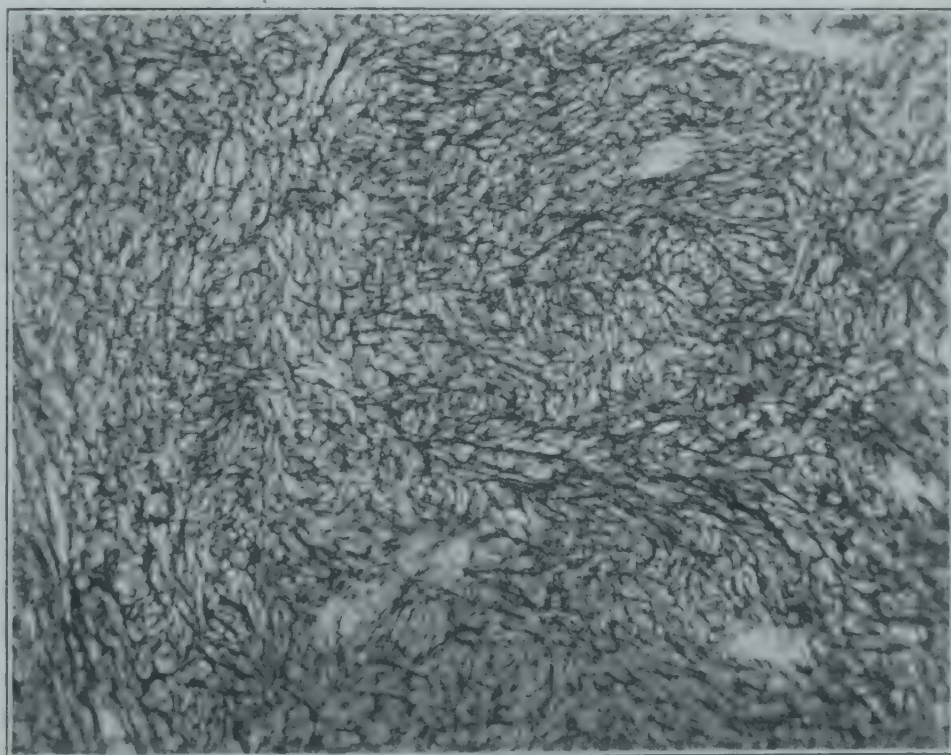


Fig. 89.—Leiomyosarcoma showing great abundance of reticulum, and absence of cellular staining.

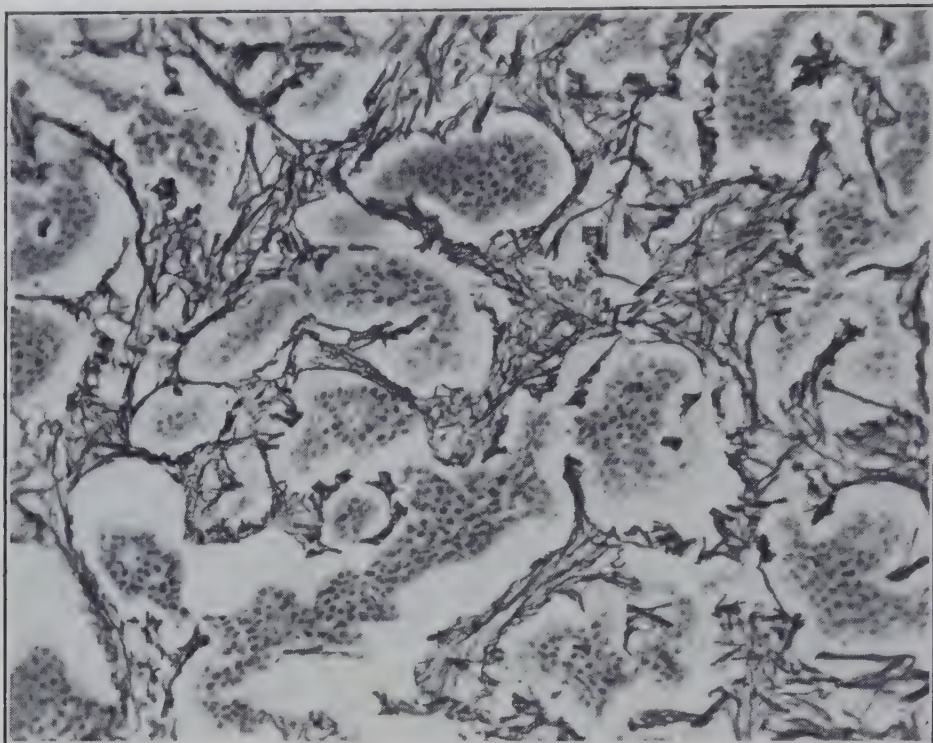


Fig. 90.—Carcinoid of liver. Note the deep stain of carcinoid cells, and complete absence of reticulum.

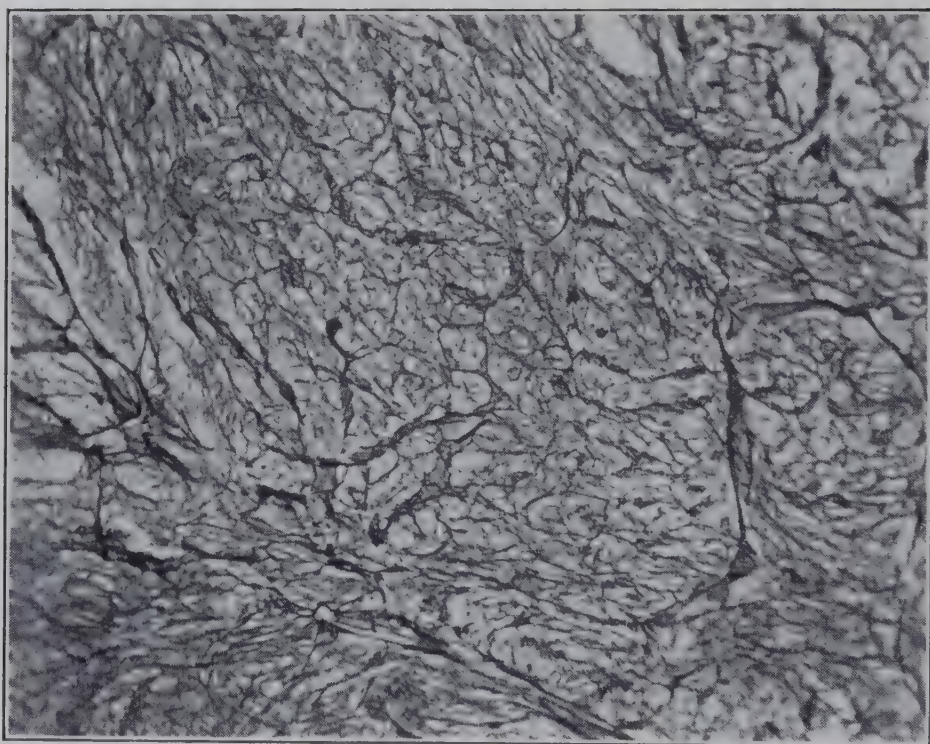


Fig. 91.—Retroperitoneal sarcoma, showing great abundance of reticulum and absence of cellular staining.

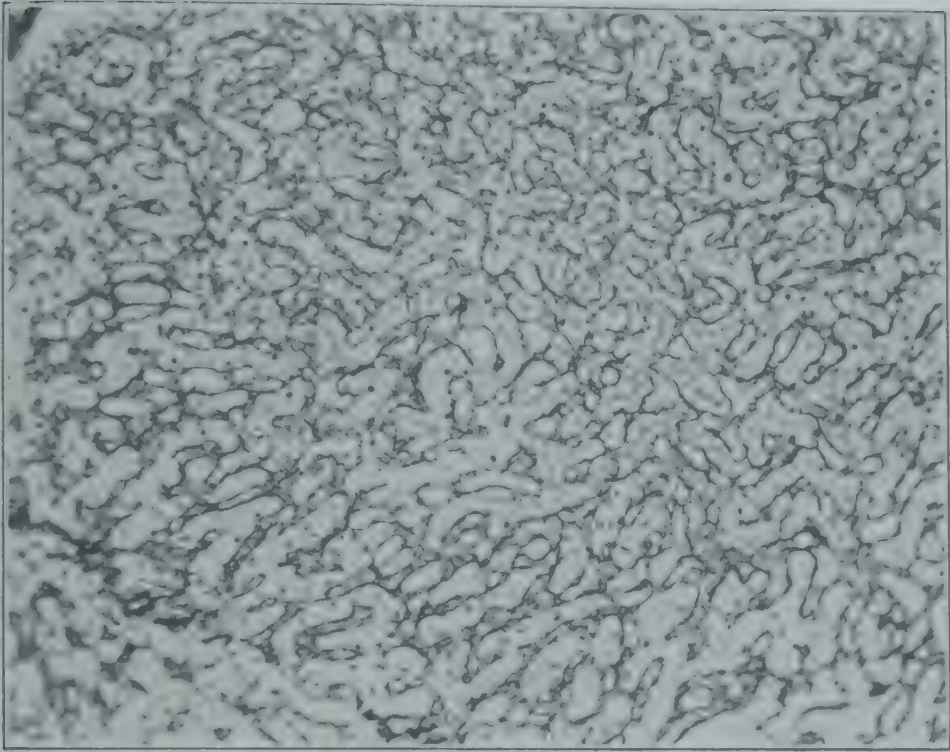


Fig. 92. Liver section showing normal reticulum.

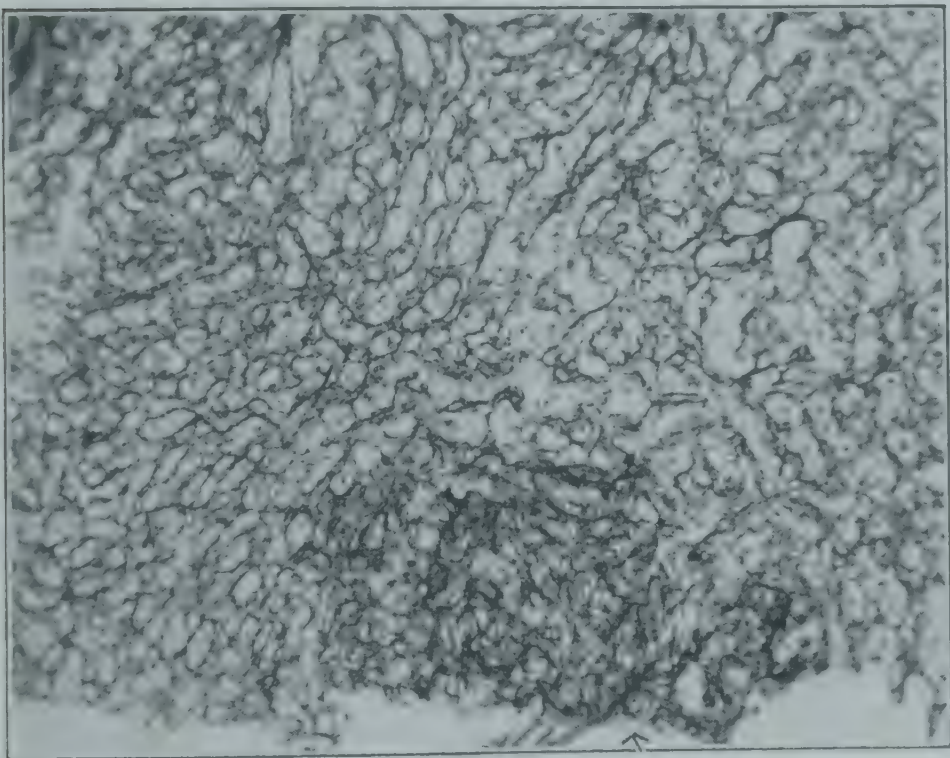


Fig. 93.—Leucemia of liver, showing abnormal amount of reticulum (arrow).

8. Wash in distilled water and place in a 20 per cent warm formaldehyde solution for 3 minutes (for reducing).

9. Wash in distilled water and place in 0.5 per cent gold chloride solution for 3 minutes.

10. Wash in distilled water and place in 5 per cent sodium thio-sulfate solution for 5 minutes.

11. Transfer to glass slide and dehydrate with 2 applications of anhydrous isopropanol or absolute ethyl alcohol, blot twice and dip in thin celloidin, blow over the surface to dry section and blot again.

12. Dehydrate twice with anhydrous isopropanol or absolute ethyl alcohol and apply creosote-xylol mixture for 3 minutes (creosote 1 part, xylol 2 parts).

13. Clear in xylol for 3 minutes and mount in Permunt or gum dammar.

For the demonstration of reticulum and collagen fibers numerous methods have been devised but unfortunately most of the methods require paraffin embedding. The paraffin method not only delays the results but may in some way obscure some of the reticulin fibers which might be demonstrated if the frozen technic were used.

Figs. 86 to 93 will serve to clarify the preceding remarks.

ELASTIC FIBER STAINS

Weigert's Elastic Fiber Stain

Fix tissues in formaldehyde or alcohol. Zenker's fixed tissues stain slowly, and there is greater tendency to diffuse coloring of collagen fibrils. Embed tissues in paraffin or celloidin, or cut frozen sections from formaldehyde-fixed tissues.

1. Stain sections 20 minutes to 1 hour in the solution prepared as follows:

Mix and dissolve 2 c.c. carbolic acid and 1 Gm. basic fuchsin in 100 c.c. distilled water, and bring the mixture to boil. When boiling, gradually add 12.5 c.c. of 30 per cent ferric chloride. A precipitate forms, and to render the precipitate more granular, continue the boiling at least 5 minutes. Cool and filter. Wash the precipitate on the filter paper with distilled water until no more color is seen. Dry the precipitate in the paraffin oven at 55° C. and keep this dry powder in a bottle for making up the staining solution.

Take 0.75 Gm. of dry powdered precipitate and dissolve in 100 c.c. of 95 per cent alcohol and boil the mixture in a flask, plugged with cotton wool, in a water bath for 20 minutes. Then cool, filter, and add 2 c.c. concentrated hydrochloric acid.

2. Wash off in 95 per cent alcohol.

3. Blot with filter paper and add xylol quickly.

Repeat the blotting, followed by xylol two or three times, until the section is perfectly cleared.

4. Mount in gum dammar.

The elastic fibers appear dark blue, almost black, on a clear background. The nuclei can be stained red with carmine, before or after staining of the fibers. Carmine stain is hard to obtain after Zenker's fixation. A light nuclear stain, with some alum hematoxylin, after the fibers have been colored, is preferable. This is a very useful and permanent stain.

Krajian's Method for Staining Elastic Fibers

A rapid method for elastic tissue, connective tissue, fibrin, and amyloid, employing Congo red (Arch. Path., Sept., 1934):

1. Fix tissues in 10 per cent formaldehyde 24 hours or longer.
2. Cut frozen sections about 10 microns.
3. Place in 2 per cent aluminum chloride solution for 5 minutes.
4. Wash thoroughly in tap water.
5. Place in the following stain for 10 minutes:

Congo red solution, aqueous 4 per cent	8 c.c.
Glycerin (C.P.)	2 c.c.

6. Wash quickly in tap water.

7. Transfer to 1 per cent aqueous potassium iodide solution for 10 seconds (rotating section in the solution).

8. Wash thoroughly in tap water.

9. Place sections in the following stain for 5 to 10 minutes:

Resorcinol	3.0 Gm.
Aniline blue (soluble in water)	1.5 Gm.
Orange G	2.5 Gm.
Phosphomolybdic acid	1.0 Gm.
Distilled water	100 c.c.

10. Wash thoroughly in tap water in a large basin. Float sections onto slides and blot with filter paper.

11. Dehydrate in absolute alcohol for 2 minutes, changing alcohol twice (dropping bottle method).

12. Clear 2 minutes in oil origanum.

13. Clear in pure xylol.

14. Mount in gum dammar.

This method gives a colorful, well-differentiated preparation that keeps well when protected from direct exposure to light.

Elastic fibers appear bright red; fibrin and connective tissue, dark blue. Red blood cells are colored orange yellow.

Sections embedded in paraffin may be used satisfactorily, but require longer staining periods. The staining technic is the same as for frozen sections, except that it requires 30 minutes' staining in Congo red solution and 30 minutes in the aniline blue solution.

If nuclear staining is desired in addition to differentiation of elastic tissue, stain sections in Harris' hematoxylin, after Step 6, for 2 minutes. Wash, dehydrate, and clear in oil origanum and xylol.

✓ **MacCallum's Modification of Verhoeff's Elastic Tissue Stain**

1. Fix tissues in 10 per cent formaldehyde for 24 hours or longer.
2. Cut frozen or paraffin sections.
3. Stain in the following solution for 30 to 60 minutes:

Hematoxylin, 10 per cent in absolute alcohol (the usual ripened solution) -----	2 c.c.
Ferric chloride, 10 per cent aqueous solution -----	3 c.c.
Sodium iodide, 10 per cent aqueous solution -----	5 c.c.
Alcohol, 50 per cent -----	50 c.c.

4. Wash in tap water.
 5. Differentiate for a few seconds in 1 per cent ferric chloride solution.
 6. Wash in water.
 7. Dehydrate completely in absolute alcohol.
 8. Clear in xylol.
 9. Mount in gum dammar.
- Elastic fibers assume a black color.

Krajian's Modification of MacCallum's Elastic Tissue Stain

1. Fix tissues in 10 per cent formaldehyde for 24 hours or longer. In emergency, fix in paraffin oven at 56° C. for 15 to 30 minutes.
2. Cut frozen or paraffin sections in usual manner.

4. Stain frozen sections 3 to 5 minutes, and paraffin sections 7 to 10 minutes, in the following solution:

Hematoxylin crystals	-----	3 Gm.
Ferric chloride	-----	3 Gm.
Ferric ammonium sulfate	-----	3 Gm.
Potassium iodide	-----	3 Gm.
Alcohol, 95 per cent	-----	25 c.c.
Distilled water	-----	25 c.c.

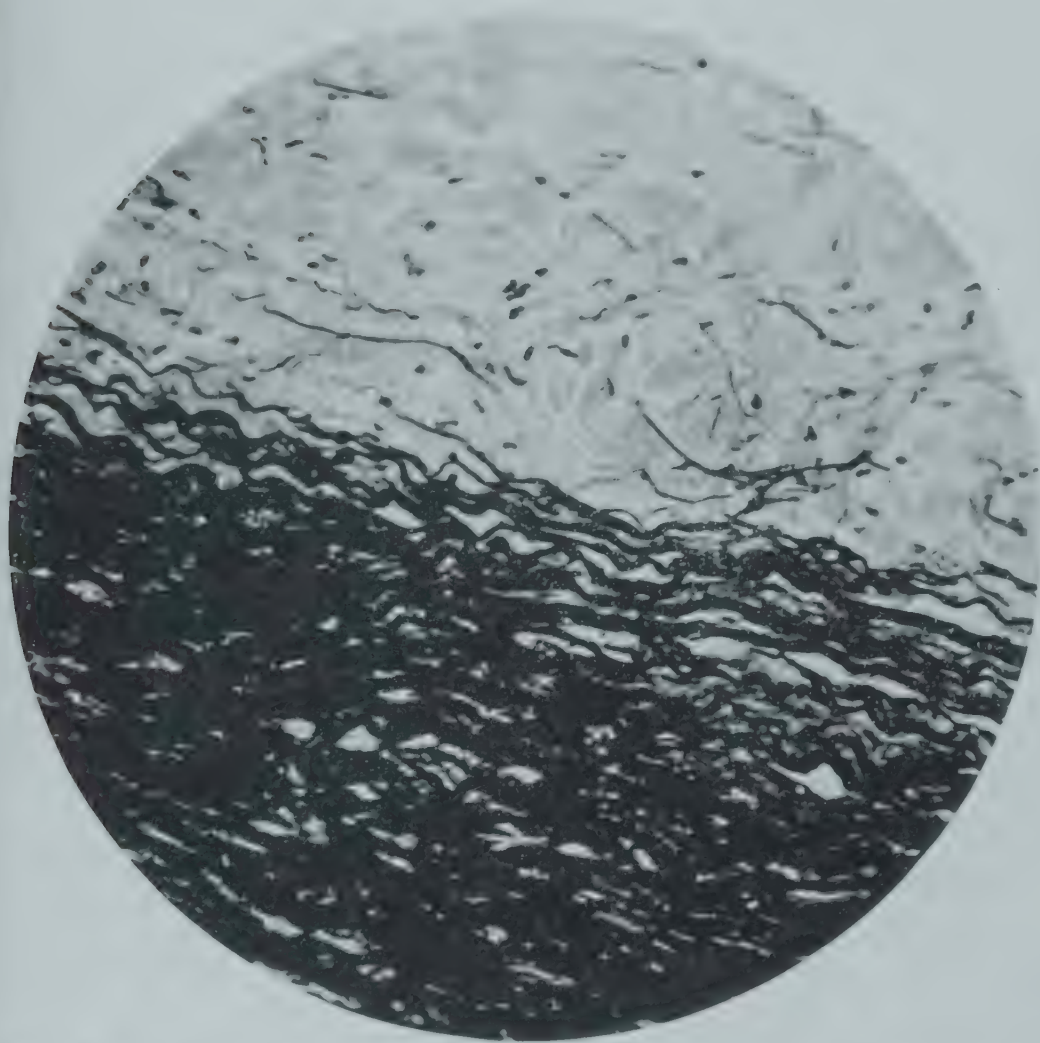


Fig. 94.—Section of aorta from a case of syphilitic aortitis, stained with Krajan's elastic tissue stain. Elastic fibers stain blue black; degenerated area remains unstained. Frozen section.

Dissolve hematoxylin crystals in alcohol, and other reagents in water, then mix the two solutions together.

4. Wash in tap water.

5. Differentiate for a few seconds in 1 per cent acid alcohol until background begins to clear. Examine under low-power lens of microscope.

6. Wash in water.
7. Treat with 2 per cent aqueous solution of zinc sulfate for 2 minutes.
8. Wash in water.
9. Dehydrate completely in absolute alcohol or isopropanol.
10. Blot and clear in xylol.
11. Mount in gum dammar.

Treatment with van Gieson solution for a few seconds after Step 4 gives a combination of elastic and connective tissue stain. Prolonging the treatment in van Gieson solution will affect the elastic fibers. For this reason sections should be examined under microscope for desired results.

FIBRIN STAINS

In fresh specimens, fibrin swells and dissolves rapidly on treatment with 1 per cent glacial acetic acid.

Weigert's Fibrin Stain

Weigert's fibrin stain is a modified Gram-Weigert method. It is by no means specific for fibrin, for it sometimes stains hyaline and coarse muscle fibers also.

1. Fix tissues in alcohol or formaldehyde.
2. Stain sections on slides in 1 per cent aqueous eosin solution or lithium carmine for 5 minutes.
3. Wash in water and stain in Sterling's gentian violet, 10 minutes for paraffin sections and 3 minutes for frozen sections.
4. Wash with normal salt solution.
5. Gram's iodine solution for 3 minutes.
6. Wash with water.
7. Blot and destain in aniline oil-xylol mixture (equal parts) until section is free from excess violet color.
8. Clear in 2 changes of pure xylol, blotting between changes.
9. Mount in gum dammar.

The fibrin, gram-positive bacteria, and fungi are stained blue. The nuclei take red stain.

This is a rapid and useful method to demonstrate fibrin in tissue.

Krajian's Stain for Fibrin

This is a combination stain to demonstrate elastic tissue, connective tissue, fibrin, and amyloid, employing Congo red. It has been found especially useful to demonstrate fibrin in lungs from unresolved pneumonia. (See index under Elastic Tissue Stain.)

Mallory's Iron Chloride-Hematoxylin for Fibrin

1. Prepare frozen or paraffin sections. Stain sections on the slide for 5 minutes in a 10 per cent aqueous ferric chloride solution.
 2. Drain and blot, then pour on a few drops of freshly prepared 1 per cent aqueous solution of hematoxylin. If all the hematoxylin is precipitated by the excess ferric chloride, pour off the solution and add a fresh supply. In 3 to 5 minutes the sections will be colored bluish black.
 3. Wash in water.
 4. Decolorize and differentiate in a 0.25 per cent aqueous solution of ferric chloride. The sections should be kept constantly moving in the solution. The differentiation will be complete in a few seconds to 1 minute or more.
 5. Wash in water.
 6. Dehydrate in 95 per cent alcohol, follow with absolute alcohol.
 7. Clear in oil origanum, 3 to 5 minutes.
 8. Clear in 2 changes of xylol, 3 minutes each.
 9. Mount in gum dammar.
- Nuclei take a sharp, permanent, dark blue stain; fibrin, grayish to dark blue stain.

AMYLOID STAINS

Amyloid is a firm translucent albuminous substance. In fresh material, Gram's iodine turns amyloid a deep brown. Add a little dilute acetic acid to the preparation before treating with iodine solution. Treated first with iodine, then with concentrated sulfuric acid, the color of amyloid material will change from brown to blue.

Krajian's Amyloid Stain

(Modified Bennhold Method)

1. Stain unmounted frozen sections in Harris' hematoxylin about 15 seconds and wash thoroughly in tap water until they are light blue.

2. Transfer to the following stain for 15 minutes:

Congo red solution, 4 per cent aqueous	-----	8 c.c.
Glycerin (C.P.)	-----	2 c.c.

3. Wash in tap water.

4. Differentiate in a 2 per cent aqueous solution of sodium cyanide for a few seconds, and wash thoroughly in tap water.

5. Float sections onto slides and allow to drain thoroughly (not dry).

6. Mount in warm glycerin jelly.

Amyloid is stained red and is beautifully differentiated from surrounding tissue. The clearing of the tissue does not in any way interfere with the amyloid stain, and the picture is further enhanced by the hematoxylin nuclear stain. The elastic fibers also are stained red, but are of a darker hue and may be easily differentiated from amyloid.

Of the number of staining methods for amyloid, this one stands out for its brilliant staining and permanent keeping without fading.

Iodine and Sulfuric Acid Reaction

1. Stain sections quickly in dilute Lugol's solution.
2. Treat with dilute sulfuric acid (5 per cent) on the slide.

The change of color from red to blue usually occurs within a few minutes, but occasionally does not take place at all.

This is not a dependable method.

Reaction With Methyl Violet

1. Stain frozen sections in a 1 per cent aqueous methyl violet 3 to 5 minutes. Rinse in water.
2. Differentiate in a 1 per cent aqueous acetic acid.
3. Wash thoroughly in tap water to remove all traces of acid.
4. Mount in glycerin jelly.

The amyloid is stained violet red and the tissue blue. The stain will keep only for a few hours and soon fades.

HYALINE STAINS

Hyaline degeneration takes place in the liver, in renal epithelium, in lymph nodes, and in some malignant tumors. Chemically, very little is known regarding it, other than that it is protein in nature.

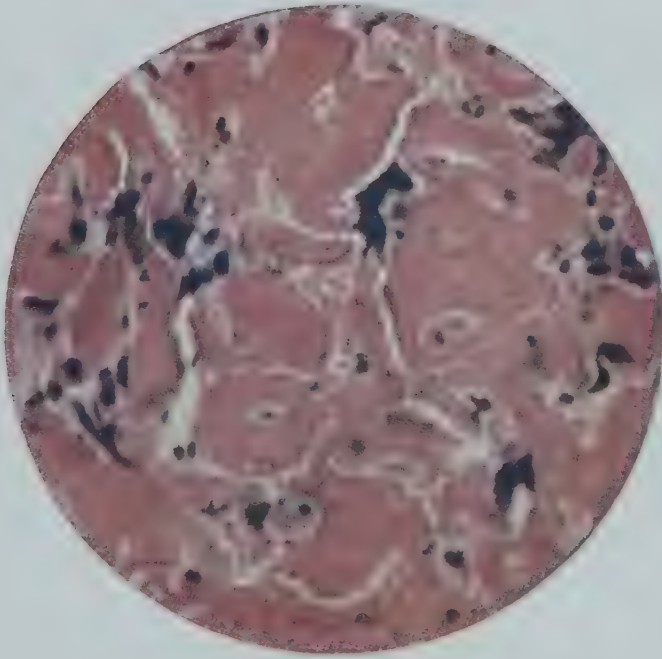


PLATE III

Section of liver stained with Krajan's amyloid stain. Amyloid, rose color; nuclei, dark blue. Frozen section.

Magnification 250 diameters. Original on Kodachrome. Made with Bausch & Lomb 8.3 MM apochromatic objective of 0.65 N.A. and 7.5X compensating eyepiece.

In fresh specimens it resists dilute acids, alkalis, and alcohol, and is not affected by water. It does not give a brown reaction when treated with iodine, nor does it turn blue after treatment with sulfuric acid. In sections it takes an uneven blue color when stained with Krajian's elastic and connective tissue stain.

Kuhne's Modified Technic

1. Fix tissues in formaldehyde, Zenker's fluid, or alcohol.
2. Stain frozen, paraffin, or celloidin sections on the slide for 3 minutes in the following freshly mixed solution:

Saturated solution of crystal violet in absolute alcohol	----	9 parts
Ammonium carbonate, 1 per cent aqueous solution	-----	1 part

3. Wash in tap water.
4. Treat with Lugol's solution for 2 minutes.
5. Rinse rapidly in tap water.
6. Differentiate in a saturated solution of fluorescein in absolute alcohol, until the hyaline material stains in various shades of blue, and the other tissues decolorize.
7. Rinse in tap water.
8. Counterstain for 2 minutes in 1 per cent aqueous solution of neutral red.
9. Rinse in tap water.
10. Dehydrate rapidly in 90 per cent alcohol and then absolute alcohol, controlling the degree of differentiation of the neutral red under the microscope.
11. Clear in xylol and mount in gum dammar.

The introduction of neutral red into this method gives an extremely transparent nuclear stain. The hyaline substance stains in shades of blue; the older the hyaline the darker the blue.

This is a rapid and useful stain.

Russell's Carbolfuchsin Method

1. Fix tissues by one of the standard fixatives. Cut frozen, paraffin, or celloidin sections and treat them on the slide as follows:
2. Stain in carbolfuchsin for 10 to 30 minutes.
3. Rinse in tap water.
4. Pass rapidly through absolute alcohol ($\frac{1}{2}$ to 1 minute).

5. Differentiate and counterstain for a few minutes in the following solution:

Iodine green -----	1 Gm.
Phenol, 5 per cent aqueous solution -----	100 c.c.

6. Rinse rapidly in absolute alcohol.

7. Clear in xylol and mount in gum dammar.

Hyaline substance stains bright red; nuclei, light green.

This is a very useful stain.

Weigert's Fibrin Stain

(Gram-Weigert Method)

Weigert's fibrin method stains hyaline material blue black. (See Gram-Weigert method.) Krajian's new connective tissue and hyaline stain, applicable to frozen and paraffin sections of formaldehyde-fixed tissue, stains hyaline bright red. (See index for technic.)

GLYCOGEN

Glycogen is a carbohydrate occurring in cells and nuclei, either diffusely or more commonly in large or small granules. It is transparent in appearance and water soluble, but can be precipitated by absolute alcohol.

The tissues for the detection of glycogen should be as fresh as possible, since glycogen may be transformed into sugar after death. Tissues removed from the body should first be dried with a towel and immediately placed in absolute alcohol. After fixation for 1 hour, they should be transferred into fresh absolute alcohol.

There are two good methods for staining glycogen: Langhan's iodine method and Best's carmine method.

Langhan's Iodine Stain

1. Fix thin blocks of tissue in absolute alcohol, clear in xylol, and embed in paraffin.

2. Cut paraffin sections, dissolve paraffin in pure xylol, and then absolute alcohol.

3. Stain sections in Lugol's solution for 5 to 10 minutes.

4. Dehydrate in 1 part of tincture of iodine to 4 parts of absolute alcohol. Wipe around section rapidly.

5. Clear and differentiate in oil origanum.

6. Wash in xylol to stop overdifferentiation.

7. Mount in gum dammar.

The standard method is to mount the section in oil origanum or gum dammar, but no matter how the sections are mounted, the stain tends to fade. Glycogen is stained brownish red; the nuclei, if stained in hematoxylin, are greenish.

This is a rapid stain, but not permanent.

Best's Carmine Method Modified

(Krajian)

Fix tissues in absolute alcohol. Embed in either paraffin or celloidin. Celloidin is preferable, since it prevents the glycogen from dissolving in water. If paraffin sections are used they must, after deparaffinization, be dipped twice in a thin celloidin (commonly used for attaching frozen sections). The celloidin or modified paraffin sections are then stained in the following manner:

1. Stain sections deeply in Harris' or Delafield's hematoxylin.
2. Destain if necessary and wash thoroughly in tap water until blue.
3. Mordant in 5 per cent alcoholic solution of benzoic acid for 5 minutes.
4. Without washing add an equal volume of carmine solution, mix well, and let stand for 5 minutes.

The staining solution:

Carmine	2 Gm.
Potassium carbonate	1 Gm.
Potassium chloride	5 Gm.
Distilled water	60 c.c.

Warm this solution for a few minutes, cool, and add 20 c.c. concentrated ammonium hydroxide. The solution must be kept in a dark cool place, and should be filtered before use. It is unstable and keeps only one month or less.

5. Differentiate in the following solution, for 1 minute or more, dipping section in and out, until no more pink color is seen:

Alcohol, 95 per cent	40 c.c.
Alcohol, methyl	20 c.c.
Distilled water	50 c.c.

6. Dehydrate in absolute alcohol or anhydrous isopropanol, then in xylol-acetone solution (1 part acetone to 4 parts xylol).

7. Clear in xylol and mount in gum dammar.

(Glycogen stains red; nuclei, blue.

This is an excellent and permanent method for glycogen.

Treatment of pale-staining sections with 1 per cent aqueous basic fuchsin, acidified with 0.5 per cent hydrochloric acid, will aid to brighten the glycogen stain.

MUCIN

Mucin is an intercellular secretory product, having some proteid substance. It is soluble in weak alkalies, and is precipitated by 1 per cent acetic acid solution, forming threads or granules.

Hoyer's Thionine Stain

Tissues may be fixed in one of the standard fixatives, but saturated aqueous solution of mercuric chloride gives the best results.

1. Stain paraffin sections after freeing them from paraffin, for 10 to 15 minutes, in the following solution:

Thionine, saturated aqueous solution	-----	2 drops
Distilled water	-----	5 c.c.

2. Dehydrate rapidly in absolute alcohol.

3. Clear in xylol.

4. Mount in gum dammar.

Mucin stains purple to red; other tissues, in shades of blue. However, mast cell granules, ground substance of cartilage, and jelly of umbilical cord also stain purple by this method. Do not treat the mercury-fixed sections in iodine, because they will spoil the stain.

This is a useful and permanent method.

Mayer's Mucicarmine Stain Modification

(Krajian)

Tissues may be fixed in 10 per cent formaldehyde or Zenker's fluid, and frozen or paraffin sections made.

1. Stain sections deeply in Harris' or Delafield's hematoxylin for about 3 minutes.

2. Wash in tap water until blue.

3. Stain for 10 to 15 minutes in the following solution:

Carmine -----	1 Gm.
Aluminum chloride -----	0.5 Gm.
Distilled water -----	25 c.c.
Alcohol, 95 per cent -----	25 c.c.

Boil and dissolve the aluminum chloride in 25 c.c. distilled water and add the carmine slowly, stirring the mixture constantly for 3 minutes. Cool, filter, and add 25 c.c. of 95 per cent alcohol.

4. Rinse and treat with martius yellow for a minute.

5. Dehydrate in several changes of absolute alcohol or isopropanol, blot in filter paper.

6. Clear in pure xylol and mount in gum dammar.

Mucin is stained pink. Cartilage cells also are stained pink, though some claim this is a specific stain for mucin.

This method is applicable to frozen sections, is easy to perform, and gives brilliant results.

Mucin Stain

Carbolfuchsin Method

(Krajian)

Fix tissues in 10 per cent formaldehyde.

1. Prepare frozen or paraffin sections in usual manner.

2. Stain in the following solution for 5 minutes:

Carbolfuchsin (Ziehl-Neelsen) -----	15 drops
Distilled water -----	15 drops
Acetic acid, glacial -----	3 drops
Formaldehyde, 40 per cent -----	3 drops

3. Wash in tap water.

4. Differentiate with picric acid-alcohol mixture (equal volume of saturated aqueous picric acid and 95 per cent alcohol) for 5 to 10 seconds, agitating the slide during this period.

5. Wash thoroughly in tap water.

6. Dehydrate in isopropanol or 95 per cent alcohol for 1 minute, and follow with anhydrous isopropanol or absolute alcohol.

7. Blot dry and clear in xylol for 2 minutes.

8. Mount in gum dammar.

Mucin assumes magenta, and sometimes pale lilac color; nuclei, reddish brown.

The differentiation (Step 4) is very important, and should be controlled under microscope for uniform results.

The staining solution is not permanent; therefore, it should be prepared fresh before use.

Pseudomucin

Pseudomucin is dissolved in water, and when treated with 95 per cent alcohol, is precipitated in threadlike masses, which are again soluble in water.

Pseudomucin is not affected by acetic acid. It is found in certain ovarian tumors.

FAT AND LIPOID STAINS

Fats and lipoids are chemically related substances. They occur in the human body under normal and diseased conditions, generally in mixtures, rarely in pure form. The different fats and lipoids are detected by chemical methods, and large numbers of them may be recognized in color reactions by some staining method.

Fresh unfixed fat globules are soluble in ether, chloroform, alcohol, xylol, benzine, and other fat solvents. For this reason, fixing fluids should be free from these substances. Formaldehyde fixes fat-containing material, but does not dissolve its fat globules.

The following important types of fats and lipins are found in the body: true fats, fatty acids, oils, soaps, cholesterins, cholesterols, waxes, and myelins.

✓ **Scharlach R Stain for Fat**

(Krajian's Modification)

The most commonly used stain for fat is scharlach R (scarlet red). Sudan III was used originally, but it is less brilliant.

1. Fix tissues in 10 per cent formaldehyde and cut frozen sections in usual manner.

2. Pass the section quickly through 70 per cent alcohol.

3. Place for 3 minutes in saturated scharlach R solution, prepared as follows:

Scharlach R	-----	5 Gm.
Acetone, pure	-----	50 c.c.
Alcohol, 70 per cent	-----	50 c.c.
Benzoic acid	-----	1 Gm.

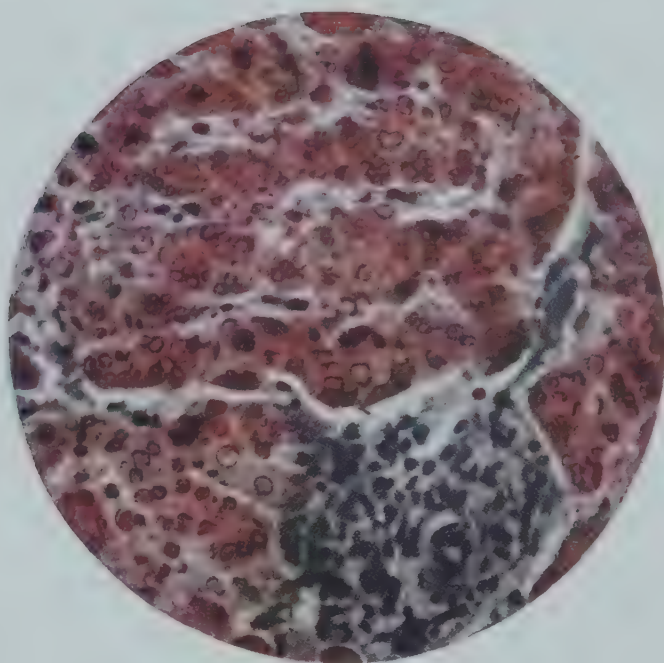


PLATE IV

Section of liver stained with scharlach R by Krajian method. Fat globules, brilliant red; nuclei, dark blue. Frozen section.

Magnification 250 diameters. Original on Kodachrome. Made with Bausch & Lomb 8.3 MM apochromatic objective of 0.65 N.A. and 7.5X compensating eyepiece.

Use only clear portion to prevent forming precipitate.

4. Wash quickly in 70 per cent alcohol.

5. Rinse in tap water.

6. Nuclear stain in Harris' hematoxylin for 30 seconds and wash in water.

7. Differentiate in 2 per cent sodium cyanide solution (aqueous) for 10 to 15 seconds.

8. Wash in water, transfer to slide, and drain off water.

9. Mount in warm glycerin jelly.

The addition of benzoic acid to the staining solution materially intensifies the resulting color and prevents deterioration. True fats stain intensely; cholesterins stain less intensely. This is a rapid and permanent stain and is highly recommended.

Osmic Acid Stain for Fat

1. Fix thin slices of tissue, not over 2 mm. thick, in Flemming's solution for 1 to 3 days.

Osmic acid, 2 per cent solution ----- 4 c.c.

Chromic acid, 1 per cent solution ----- 15 c.c.

Glacial acetic acid ----- 1 c.c.

2. Wash under running water for from 6 to 12 hours.

3. Dehydrate in 80 per cent, 95 per cent, and then absolute alcohol, about 2 hours in each.

4. Clear in chloroform. (Do not use ether, xylol, or toluol for clearing, because they dissolve the fat.) Embed in paraffin.

5. Cut sections and dissolve the paraffin with xylol.

6. Mount in chloroform balsam.

Fat globules stain black to dark gray; lipoidal granules, brownish.

This is a useful and permanent method.

Osmic Acid Method for Frozen Sections

1. Fix tissues in 10 per cent formaldehyde for 24 hours or longer.

2. Cut frozen sections.

3. Place in a 1 per cent aqueous solution of osmic acid for 1 to 2 hours.

4. Wash in several changes of tap water for 10 minutes.

5. Place in absolute alcohol or anhydrous isopropanol for 15 minutes to obtain the secondary staining of fat not yet colored.

6. Wash in tap water.

7. Mount in glycerin jelly.

Fat globules assume a brown-black color. This is a useful fat stain for frozen sections.

✓ Nile Blue Sulfate Stain for Fat

(Lorraine Smith)

1. Fix tissues in formaldehyde and cut frozen sections.
2. Stain in a saturated aqueous solution of Nile blue sulfate for 5 to 10 minutes.
3. Wash in water.
4. Differentiate in 1 per cent glacial acetic acid solution in water until the blue color ceases to diffuse from the section.
5. Wash thoroughly in a large basin of water.
6. Mount in glycerin jelly.

This stain has valuable properties. It gives a double stain, coloring neutral fats red; cholesterin ester and cholesterin fatty acids, light red; cerebrosides, light blue; and fatty acids and soaps, deep blue.

Stain for Lipoids

(Ciaccio Method)

1. Fix thin blocks of tissue for 2 days in the following solution:

Potassium bichromate, 5 per cent solution	-----	80 c.c.
Formaldehyde (Commercial, 40 per cent)	-----	20 c.c.
Glacial acetic acid	-----	5 c.c.
or formic acid	-----	4 to 5 drops

2. Treat in 3 per cent aqueous solution of potassium bichromate for 2 days.
3. Wash under running water overnight.
4. Dehydrate in graded alcohols, beginning with 70 per cent, and reduce the time in 95 per cent and absolute alcohol as much as possible.
5. Xylol for 1 hour.
6. Xylol and paraffin mixture for 1½ hours in the paraffin oven at 55° C.
7. Pure paraffin for 1½ hours in the paraffin oven.
8. Cut sections and fix them on the slides.
9. Dissolve the paraffin with 2 changes of xylol.
10. Wash in 2 changes of absolute alcohol.

11. Stain in a saturated solution of sudan III (95 c.c. of 85 per cent alcohol plus 5 c.c. acetone). Staining should be done in a stoppered slide jar, from 30 to 60 minutes, at 30° C.

12. Differentiate very rapidly in 50 per cent alcohol.

13. Wash in water.

14. Counterstain in Harris' hematoxylin for 5 minutes.

15. Wash in water until section assumes blue color.

16. Mount in warm glycerin jelly.

The lipoid substances appear as fine orange granules; the nuclei, blue black.

This method gives a positive stain for lipoid granules.

Staining Reactions of Various Fats After Fixation With Formaldehyde and Mercuric Chloride*

Fresh frozen unfixed tissues are highly satisfactory for staining neutral fats. The unfixed fat, in general, stains a slightly lighter color than fat fixed in formol.

Formol-fixed frozen sections are the most satisfactory for routine demonstration of fat for the following reasons: The tissue does not require extensive washing before cutting; the formol preserves the fat; the sections are easily handled, and they stain more deeply with scharlach R, sudan III, and Nile blue sulfate. Formol-fixed tissues can also be used for myelin staining. Unfortunately, probably because of the development of formic acid, formol-fixed tissues, both in the mass and in sections, may suffer partial hydrolysis of some of the lipids. This liberates free fatty acids and makes it impossible to demonstrate the presence of neutral fats with Nile blue sulfate in tissue sections made from gross specimens fixed in formol, and also causes the red color in formol-fixed preparations, previously stained with Nile blue sulfate, to be gradually dominated by blue. This change may occur within a few days with the unsaturated vegetable oils.

After fixation in mercuric chloride, as compared to formol fixation, fat stains considerably lighter with scharlach R, sudan III, Nile blue sulfate, and a more distinct deeper blue with indophenol. The preparations of Nile blue sulfate and indophenol that have been fixed in mercuric chloride are more permanent than those fixed in

*Black, Charles: J. Lab. & Clin. Med. 23: 1027 (July), 1938.

TABLE I
COMPARISON OF THE STAINING REACTIONS AFTER FIXATION WITH FORMOL AND MERCURIC CHLORIDE

FAT DYE AND FIXATION	POPPY-SEED OIL AND LIPIODOL (20%)	OLIVE OIL	LARD	BUTTER FAT	MINERAL OIL	PARAFFIN
Scharlach R Formol	Deep orange red to very deep brilliant scarlet	Deep orange red to deep brilliant scarlet	Very deep bril- liant orange red	Deep brilliant orange red	Medium orange to brilliant deep red orange	Unstained
Mercuric chloride	Medium brilliant orange red	Light brilliant orange red	Light to medium brilliant orange red	Medium brilliant orange red	Light brilliant orange	Unstained
Sudan III Formol	Brilliant deep orange to deep brilliant red orange	Bright orange to deep brilliant red orange	Medium brilliant red orange	Medium brilliant red orange	Pale orange yellow	Unstained
Mercuric chloride	Medium brilliant red orange	Light to medium brilliant red orange	Very light to medium red orange	Light brilliant red orange	Light pale orange	Unstained

Nile blue sul- fate Formol	Medium to very deep brilliant magenta	Medium to deep brilliant magenta	Medium brilliant magenta	Light to medium brilliant magenta	Bright light pink	Very light pale pink
Mercuric chloride	Light brilliant magenta	Light brilliant magenta	Very light bril- liant magenta	Very light bril- liant magenta	Very pale pink	Very light pale pink
Osmic acid Formol	Black	Black	Dark brown to black	Dark gray to black	Unstained	Light to dark gray
Mercuric chloride	Black	Black	Dark brown to black	Dark gray to black	Unstained	Light to dark gray
Indophenol Formol	Deep blue to blue black	Deep blue to blue black	Dark blue to black	Dark blue to blue black	Pale blue	Very pale blue
Mercuric chloride	Very deep blue	Purple to deep blue	Deep blue to blue black	Deep blue to black	Pale purple	Very pale blue

either Müller's fluid or formol. The necessity for thorough washing and the danger of overfixation are disadvantages of this method.

After fixation in Müller's fluid the neutral fats give beautiful results, staining very well with all the fat dyes used. The color is lighter than with formol and deeper than with mercuric chloride. No essential change is noted with osmic acid as related to this fixative. Müller's fixing fluid has the disadvantage of requiring prolonged fixation and extensive washing of the tissue. It is a good preservative of fat, but not as good as mercuric chloride, as shown by the Nile blue sulfate.

The neutral fats studied, in general, stain according to their degree of unsaturation. Poppy-seed oil, the most unsaturated of the oils studied, stains the deepest. Olive oil, lard, butter fat, and the saturated hydrocarbons, including mineral oil and solid paraffin, stain progressively lighter in the order named.

There are multiple factors involved in the staining of fat. This makes it impossible to name a specific shade or tint as uniformly characteristic of a particular fat.

Paraffin (solid) can be identified with a moderate degree of certainty by its negative reaction to scharlach R and sudan III, its pink crystalline character with Nile blue sulfate, and its gray color with osmic acid.

Mineral oil (petrolatum liquidum) can be identified by its pale to deep red orange staining with scharlach R and its negative reaction to osmic acid.

Within the limited group studied, the vegetable oils stained somewhat more deeply with all the dyes used than did the animal fats.

Differential Stains for Fatty Substances

Phosphatides.—Lecithin, myelin, and cephalin show myelin formation and in part double refraction.

Stain with: Scharlach R—Yellow red
Nile blue—Dark blue
Neutral red—Reddish

Cholesterin Fats.—Fatty acids and cholesterin show no myelin formation but marked double refraction.

Stain with: Scharlach R—Yellow red
Nile blue—Red
Neutral red—No color

Glycerin Fats.—Neutral fats, fatty acids, and glycerin show neither myelin formation nor double refraction.

Stain with: Scharlach R—Bright red
Nile blue—Reddish
Neutral red—No color

Fatty Acids.—Do not form myelin and are crystalline.

Stain with: Scharlach R—Yellow
Nile blue—Blue
Neutral red—Red

Soaps.—Swell up.

Stain with: Scharlach R—Yellow
Nile blue—Blue
Neutral red—Red

Cholesterin.—Does not swell up, shows definite crystals with broken corners.

Stain with: Scharlach R—Yellow
Nile blue—Blue
Neutral red—Red

MYELIN OR MEDULLARY SHEATH STAINS

Cerebrosins and lecithins constitute the normal myelin sheath, according to Wlassak and Mann.

Weigert-Pal Method

1. Fix tissues in 2.5 per cent potassium bichromate solution for several weeks in an incubator, or fix in 10 per cent formaldehyde first, and then treat with potassium bichromate solution.
2. Wash under running water overnight.
3. Dehydrate in graded alcohols, clear, and embed in either paraffin or celloidin.
4. Cut sections 10 to 20 microns.
5. Stain in the following lithium-carbonate-hematoxylin mixture for 24 to 48 hours.

Solution A

Hematoxylin crystals	-----	10 Gm.
Alcohol, absolute	-----	100 c.c.

This solution should be ripened for 2 or 3 weeks.

Solution B

Lithium carbonate, concentrated aqueous solution--	7 c.c.
Distilled water -----	93 c.c.

Mix these two solutions just before use, in the ratio of 1 volume of solution A to 9 volumes of solution B.

6. Wash sections in water and treat with a 0.25 per cent aqueous solution of potassium permanganate for $\frac{1}{2}$ to 3 minutes.

7. Wash in water.

8. Treat sections in the following differentiating fluid for $\frac{1}{2}$ to 3 minutes:

Oxalic acid, 1 per cent aqueous solution -----	1 part
Potassium sulfite, 1 per cent aqueous solution ----	1 part

Differentiation is complete when the white matter of the brain tissue is blue black and the gray matter practically colorless.

9. Wash sections in water and counterstain in lithium carmine or safranine if desired.

10. Dehydrate in absolute alcohol, clear in carbolxylol, xylol, and mount in gum dammar.

By this method myelin sheaths appear black; recent areas of degeneration, pale gray; old ones, colorless.

We have omitted the original Weigert method and have replaced it with Pal's modification, which is more rapid in performance and gives sharper myelin sheaths. This is a slow, but very useful method.

Spielmeyer's Method

1. Fix tissues in 10 per cent formaldehyde 24 hours or longer.
2. Wash in running water.
3. Cut frozen sections 20 to 30 microns.
4. Place in a 2.5 per cent aqueous solution of iron ammonium sulfate for 6 hours.
5. Rinse in water, then place in 70 per cent alcohol for 10 minutes.
6. Stain sections for 10 to 24 hours in the following solution:

Ripe 10 per cent hematoxylin in absolute alcohol --	5 c.c.
Distilled water -----	100 c.c.

7. Rinse sections in water.

8. Differentiate in a weaker solution of iron ammonium sulfate.

9. Wash thoroughly in tap water.

10. Dehydrate in absolute alcohol.

11. Clear in xylol and mount in gum dammar.

The hematoxylin solution must be old and should be used over and over, that is, the used portion should be filtered back into the original bottle. The staining power improves with age.

The washing in 70 per cent alcohol removes fatty substances which might interfere with even staining. Occasional irregular staining indicates that the fat has not been entirely removed. Such sections may be carried back through water to 70 per cent alcohol and again stained and differentiated.

This is a very good stain to show the fine detail of myelinated fibers.

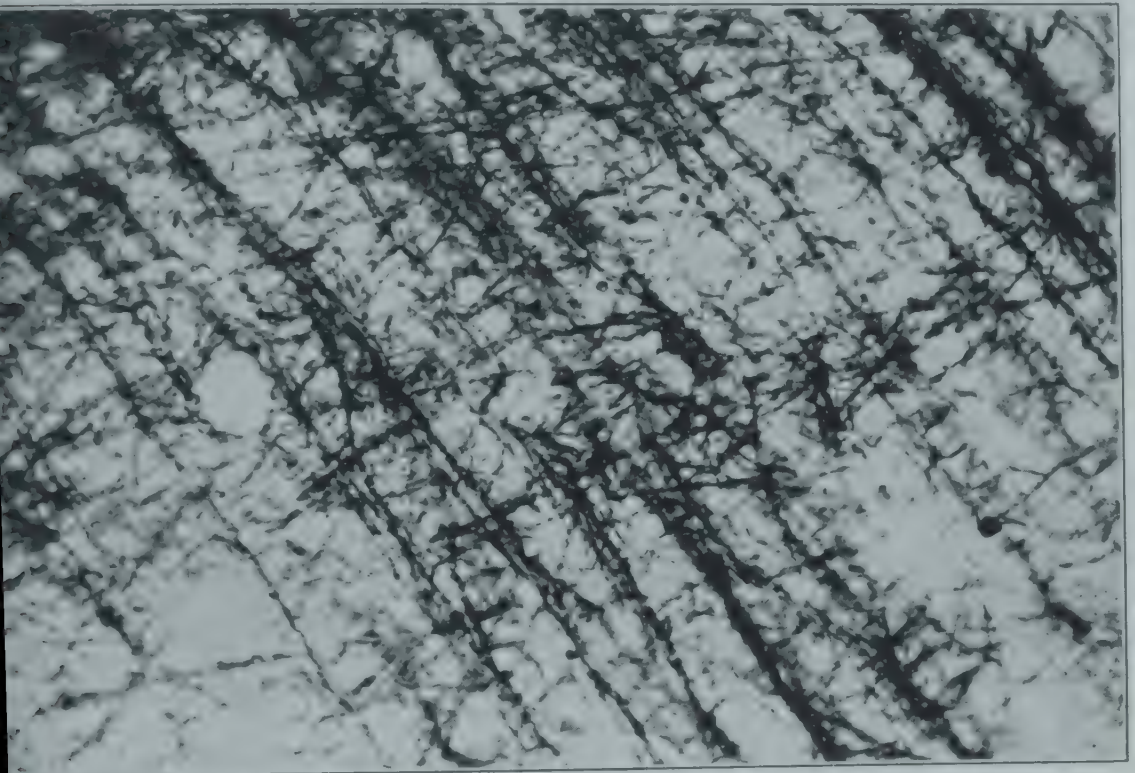


Fig. 95. High-power magnification of myelin sheath. Spielmeier's method. Frozen section.

Courville-Krajian Method

1. For rapid diagnosis on fresh tissue, boil the blocks for 1 minute in 10 per cent formaldehyde, and then leave in the same solution in a paraffin oven for 5 minutes before cutting frozen sections. When the section is not demanded in a short time, fix the tissue in 10 per cent formaldehyde for 24 hours or longer.

2. Cut frozen sections 10 microns thick. Receive sections in a large dish of tap water.

3. Draw sections onto the slides and let the excess water drain off for about a minute. Dehydrate with absolute alcohol, pouring on a few drops 2 or 3 times, each time allowing it to evaporate. Blot and dip the slide into a thin celloidin solution, fixing the section to the slide. Dip the slide into tap water for a few seconds.

4. Cover the section with hot 15 per cent aqueous solution of ferric chloride for 5 minutes. Drain the solution from the slide. Do not wash.

5. Cover section for 5 minutes with equal parts of hematoxylin solution and distilled water, heated to 60° C. This stains the section extremely black.

6. Wash thoroughly in tap water.

7. Remove excess stain in a 1 per cent aqueous solution of ferric chloride, dipping the slide in and out of the solution until the gray matter begins to appear in contrast to the dark medullary substance. This takes place in from 10 to 25 seconds.

8. Wash the slide quickly in tap water.

9. Cover the section with a 0.25 per cent aqueous solution of potassium permanganate and shake with the fingers to secure even differentiation, which takes place in about 5 seconds. This step should be controlled under the microscope.

10. Wash thoroughly in tap water.

11. Drain off excess water and dehydrate with several applications of absolute alcohol from a drop bottle. Blot between filter paper.

12. Plunge section into a mixture of equal parts of aniline oil and xylol for about 3 minutes, and then in pure xylol for another 3 minutes.

13. Mount in gum dammar.

Hematoxylin solution is prepared by dissolving 10 Gm. of hematoxylin crystals in 90 c.c. absolute alcohol, and ripening in an incubator at 37° C. for 2 or 3 weeks. This solution is stable and will keep for months.

The above method has many of the advantages of the longer methods as far as the results obtained are concerned, and yet it can be completed in 30 minutes.*

*A Rapid Method for Staining Myelin Sheaths, Arch. Path., June, 1931.

Krajan's Myelin Sheath Stain

1. Fix tissues in 10 per cent formaldehyde. Cut frozen sections 10 to 15 microns.
2. Place sections in 4 per cent Congo red in glycerin for 10 minutes (Congo red 4 per cent, 8 c.c.; glycerin, 2 c.c.).
3. Wash thoroughly in tap water.
4. Place in 2 per cent aqueous solution of potassium iodide for 2 minutes.



Fig. 96.—Tabes dorsalis. Posterior column degeneration of myelin sheath in spinal cord. Krajan's myelin sheath method. Frozen section.

5. Wash and place in 3 per cent aqueous solution of sodium cyanide for 2 minutes.
6. Wash thoroughly in tap water.
7. Place in 10 per cent aqueous ferric ammonium sulfate for 10 minutes at 55° C.
8. Wash in tap water.
9. Place in 2 per cent ripe hematoxylin solution for 5 minutes (2 c.c. ripe hematoxylin, 8 c.c. distilled water).
10. Wash in tap water.

11. Destain in 2 per cent aqueous ferric ammonium sulfate solution until section appears light blue. This takes place in 30 to 45 seconds.

12. Wash thoroughly in tap water.

13. Differentiate in 0.15 per cent aqueous potassium permanganate for 10 to 20 seconds.

14. Wash in tap water.

15. Place in 3 per cent aqueous sodium cyanide until sections flatten out and assume a light blue appearance.

16. Wash in tap water, attach to the glass slide.

17. Dehydrate in 2 changes of absolute alcohol.

18. Oil origanum, 2 minutes.

19. Pure xylol for 2 to 5 minutes.

20. Mount in gum dammar.

The above method has all the advantages of the longer methods, showing very fine details of the fibrils, and yet it can be completed in 1 hour. It is very highly recommended.

Pal-Kulschitzky's Method

1. Fix tissues in 10 per cent formaldehyde for a few days or longer and place them in the following solution for 4 to 5 days or longer at room temperature:

Potassium bichromate	-----	5 Gm.
Fluorchrome	-----	2 Gm.
Water	-----	100 c.c.

2. Place the blocks in graded alcohol for dehydration, and then in ether-alcohol mixture, thin celloidin, thick celloidin, and embed.

3. Cut sections 35 microns thick or thicker, and place in 80 per cent alcohol.

4. Stain sections at 37° C. for 24 hours in the following solution:

10 per cent ripened solution of hematoxylin in	
absolute alcohol	----- 10 c.c.

2 per cent glacial acetic acid	----- 90 c.c.
--------------------------------	---------------

(The hematoxylin solution must be at least 6 months old.)

5. Wash sections individually in tap water.

6. Place in 0.3 per cent aqueous solution of potassium permanganate for about 1 minute or longer.

7. Wash in distilled water.

8. Differentiate in the following solution until the gray matter is colorless:

Oxalic acid, 1 per cent aqueous solution

Potassium sulfite, 1 per cent aqueous solution

For use, mix equal parts.

9. Wash in tap water, in which the sections can be kept for any length of time. In case the differentiation is unsuccessful, the sections may be carried back from water into potassium permanganate solution, and from there again to water and oxalic potassium sulfite solution. This procedure can be carried out several times until the differentiation is completed.

10. Dehydrate in graded alcohols, clear in pure xylol, and mount in gum dammar.

Wolter has modified this method so that it may also be used for frozen sections. Cut frozen sections about 40 microns thick and place them in 70 per cent alcohol, then in bichromate and fluorochrome solution for 24 hours at 37° C. From the latter (the mordanting fluid) the sections are passed through 80 per cent alcohol to the hematoxylin solution, in which they are kept 24 hours at 37° C. Staining is completed as above, commencing with Step 5.

This method usually yields satisfactory results and is valuable for rapid staining of myelin sheaths.

Morgan's Myelin Stain for Paraffin Sections

Morgan's method of staining myelin is useful in fresh, as well as old, formol-fixed material. The directions are as follows:

1. Fix brain tissue in 10 per cent formaldehyde, embed in paraffin, and section.

2. Mordant sections for 2 hours in a 4 per cent aqueous solution of iron alum (ammonium ferric sulfate).

3. Stain 8 to 16 hours in well-ripened Heidenhain's hematoxylin (hematoxylin 0.5 Gm., absolute alcohol 10 c.c., distilled water 90 c.c.).

4. Destain in 2 per cent iron alum for a few minutes until the larger fiber tracts become well outlined.

5. Rinse in tap water.

6. Complete the differentiation in 0.5 per cent hydrochloric acid. In a few seconds the background becomes colorless and the fibers well differentiated.

7. Wash sections in running water, for at least an hour, to remove acid and alum.

8. If desired, counterstain with well-ripened neutral red.

9. Dehydrate, clear in xylol, and mount in gum dammar or balsam.

In successful preparations, the myelinated fibers are stained a dark blue, and the background is clear and white. For destaining with 2 per cent alum, the process should be stopped by placing the sections in tap water before all the blue of hematoxylin has been extracted from the background; and the treatment with the hydrochloric acid should be very brief and carefully watched. When the proper stage has been reached, the decolorizing is stopped by transferring the sections to tap water.

Scharlach R Stain for Demonstration of Myelin Sheath

The improved scharlach R solution, ordinarily used to demonstrate fat, may be successfully employed to bring out myelin sheath in the tissues of the central nervous system, especially in spinal cords (see "Scharlach R Stain for Fat").

XANTHYDROL METHOD FOR UREA*

Regular Method

1. Cut thin blocks of tissue, from 2 to 3 mm. in thickness. Immerse for from 5 to 6 hours in a freshly prepared solution of xanthydrol (5 Gm. of xanthydrol in 100 c.c. glacial acetic acid).

2. Wash under running water for 1 hour.

3. Fix in dilute solution of formaldehyde U.S.P. (1:10) 6 hours or longer. In urgent cases a rapid fixation with heat may be used.

4. Wash in tap water.

5. Cut frozen sections 10 microns thick.

6. Transfer to glass slide.

7. Pour several drops of dehydrated alcohol over the section from a drop bottle and blot. Repeat this process once more.

8. Cover with thin pyroxylin (celloidin), dipping the slide in a wide-mouthed bottle containing pyroxylin.

9. Fix to the slide by blowing breath over the section.

*Krajian's Modification of Oestreicher's Technic, *Arch. Path.* 21: 96 (Jan.), 1936.

10. Stain in a 1 per cent aqueous solution of eosin for several minutes.
11. Wash in tap water.
12. Dehydrate in 3 changes of dehydrated alcohol (drop bottle method).
13. Place in carbolxylol for 2 minutes.
14. Clear in 2 changes of pure xylol, 1 minute in each.
15. Mount in dammar.

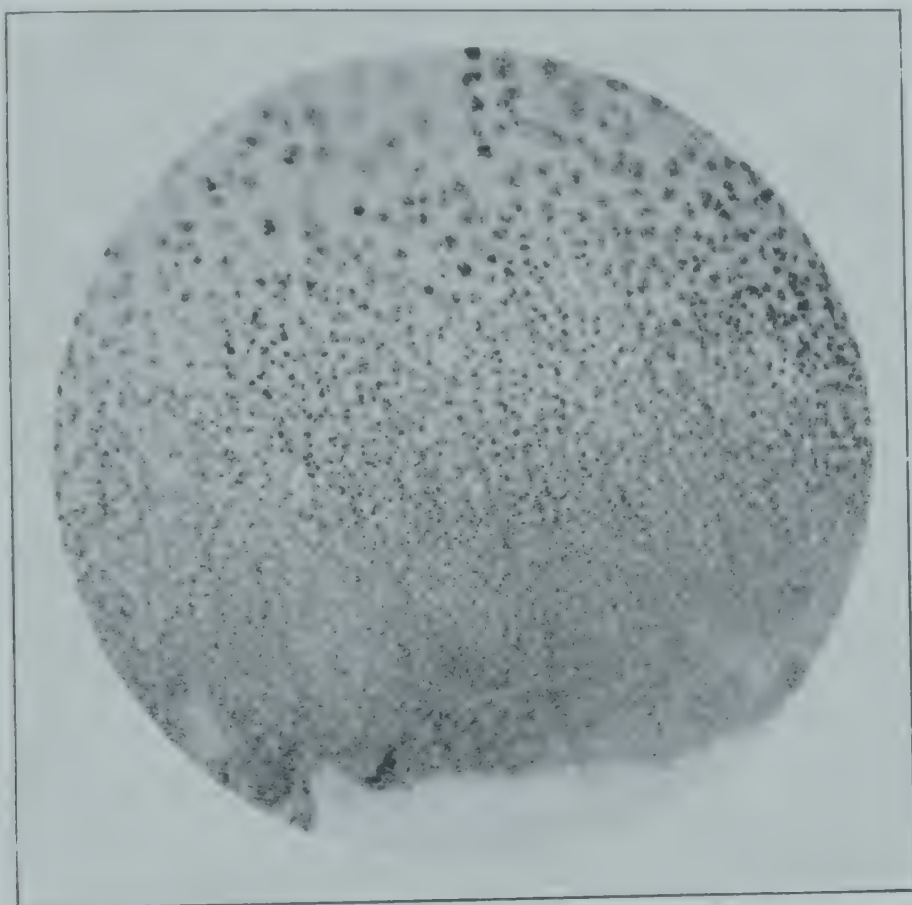


Fig. 97. Xanthhydrol method for urea by regular method (6 hours' fixation). Low-power magnification. (Krajan method.)

Owing to the high diffusibility of urea, the tissue must be kept from contact with any fluid previous to its immersion in xanthhydrol. One should especially avoid contamination with urine during the autopsy. The blocks must be very thin, since the rate of penetration by the xanthhydrol-acetic acid solution is very slow. The use of hematoxylin to stain the nuclei has a tendency to disturb the crystals; accordingly it is preferable merely to stain the background with eosin.

Rapid Xanthydrol Method

1. Fix thin blocks in hot xanthydrol solution (5 per cent) 1½ hours in oven at 70° C.
2. Wash in tap water 1 to 2 minutes.
3. Fix in hot 10 per cent formaldehyde 15 minutes at 70° C.
4. Rinse in tap water.
5. Cut frozen section as superficially in the block as possible, since penetration by the xanthydrol is very shallow.
6. Stain and mount as in longer technic.

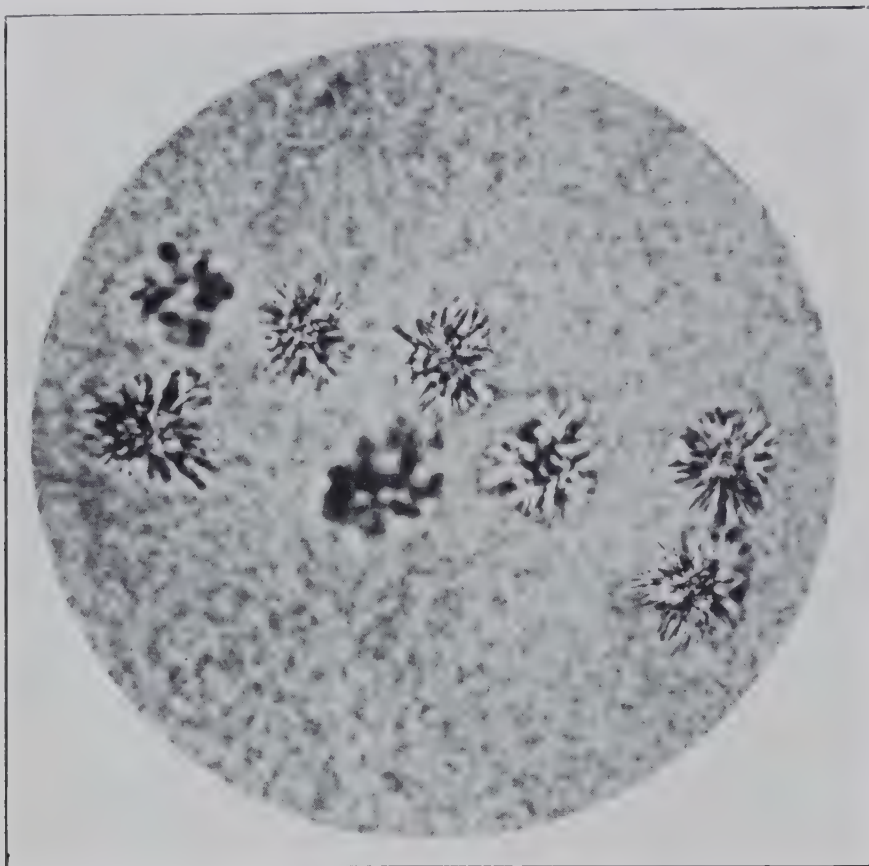


Fig. 98.—Xanthydrol method for urea. Rapid method (1½ hours' fixation in oven at 70° C.). High-power magnification. (Krajan method.)

Smear Method for Xanthydrol Test

For very rapid, approximate, or preliminary diagnosis, the following technic is used:

1. Boil very small bits of brain tissue in a test tube for 2 minutes in 5 per cent xanthydrol solution.
2. Smear tissue on glass slide.
3. Flood with absolute alcohol 2 or 3 times.
4. Stain as in other methods.

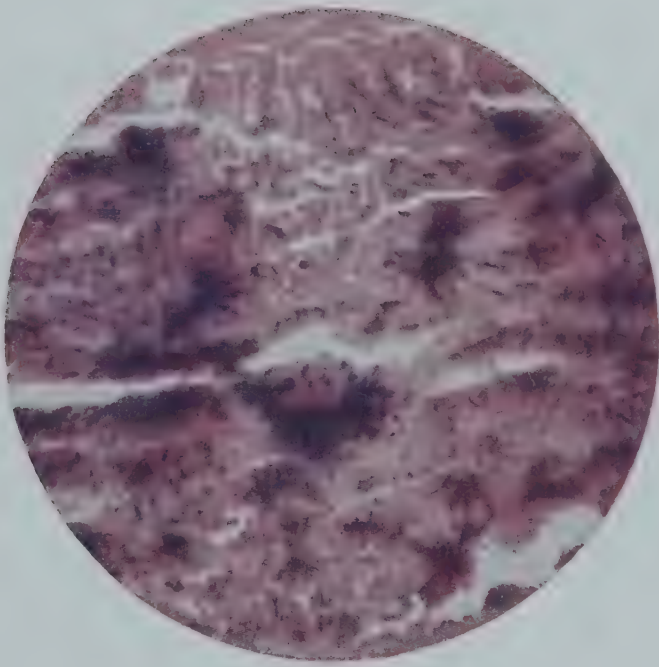


PLATE V

Actinomyces colonies in a kidney section stained by Gram-Weigert method. Actinomyces, dark violet; background, red. Frozen section.

Magnification 250 diameters. Original on Kodachrome. Made with Bausch & Lomb 8.3 MM apochromatic objective of 0.65 N.A. and 7.5X compensating eyepiece.

STAINING METHODS FOR BACTERIA, FUNGI, MALARIA, AMOEBAE, NEGRI BODIES, SPIROCHETES, COCCIDIODES, AND BLASTOMYCES

For the demonstration of gram-positive and gram-negative organisms, several methods have been devised or modified, but the Gram-Weigert stain still remains the standard and reliable method.

Gram-Weigert Method

1. Fix tissues in formaldehyde and make very thin paraffin, celloidin, or frozen sections.
2. If paraffin sections are made, remove paraffin with 2 changes of xylol, and then 2 changes of absolute alcohol.
3. Stain in 1 per cent aqueous solution of eosin for 5 minutes.
4. Wash in water.
5. Stain in Sterling's gentian violet 3 minutes for frozen sections, or 10 minutes for paraffin sections.
6. Wash off with Gram's iodine solution and flood with the same for 3 minutes.
7. Blot in fine filter paper.
8. Decolorize with several changes of equal parts of aniline oil and xylol until color ceases to wash out.
9. Clear in 2 changes of xylol, blotting after each change.
10. Mount in gum dammar.

As a counterstain, a weak carbol fuchsin or 1 per cent aqueous solution of neutral red may be employed instead of 1 per cent eosin.

With this method, gram-positive organisms and fungi stain a beautiful violet color, but gram-negative organisms are not ordinarily seen.

MacCallum's Modification of Goodpasture's Method for the Demonstration of Gram-Negative Organisms

Fix tissues in Zenker-formaldehyde solution (Helly's fluid), embed in paraffin, and make very thin sections.

1. Stain sections for 10 to 30 minutes or longer in Goodpasture's fuchsin solution:

Alcohol, 30 per cent	-----100	c.c.
Basic fuchsin	-----0.59	Gm.
Aniline oil	-----1	c.c.
Phenol crystals	-----1	Gm.

2. Wash in water.

3. Differentiate in formalin (40 per cent solution of formaldehyde); only a few seconds are required. The bright red color changes to rose.

4. Wash in water.

5. Counterstain in a saturated aqueous solution of picric acid for 3 to 5 minutes until the section becomes purplish yellow.

6. Wash in water.

7. Differentiate in 95 per cent alcohol. The red reappears and some of it is washed out, as is also some of the yellow of the picric acid.

8. Wash in water.

9. Stain in Sterling's gentian violet for 5 minutes or longer.

10. Wash in water.

11. Gram's iodine solution for 1 minute.

12. Blot dry without washing.

13. Clear in a mixture of equal parts of aniline and xylol until color ceases to wash out.

14. Clear in two changes of xylol.

15. Mount in gum dammar.

Gram-negative bacteria stain red; gram-positive bacteria, blue; tissue, red and blue; fibrin, deep blue.

This is a very useful stain to demonstrate gram-negative organisms.

Application of Gram Stain on Paraffin Sections*

(Glynn)

Paraffin sections are brought through xylol and alcohol to water in the usual manner.

1. Stain with carbol gentian violet for 2 minutes.

2. Drain but do not wash.

3. Apply Gram's iodine for 1 minute.

4. Apply acetone until no more color is removed (about 10 to 15 seconds).

5. Wash in water. Do not allow the section to dry.

6. Apply 0.05 per cent basic fuchsin in 0.05 N hydrochloric acid for 3 minutes.

7. Drain but do not wash.

8. Apply saturated aqueous picric acid for from 30 seconds to 1 minute.

*Arch. Path. 20: 896, 1935.

9. Wash in water.

10. Differentiate and dehydrate in acetone for from 10 to 15 seconds.

11. Clear in xylol.

12. Mount in balsam.

The gentian violet used is a product of the National Aniline and Chemical Company labeled "Gentian Violet (Crystal Violet)." Its dye content is 90 per cent. The color index number is 681. It has been certified by the Commission of Standardization of Biological Stains with certification number N.C. 14. The stain was prepared by triturating 1 Gm. gentian violet and 1 Gm. phenol crystals in a mortar, and then adding 10 c.c. absolute alcohol. This stock solution was diluted ten times with distilled water, allowed to stand 48 hours, and filtered before using. This particular dye is not essential to the method. Equally good results have been obtained using methyl violet 5 B Grubler and crystal violet Grubler.

The basic fuchsin employed is a product of Grubler. Further information about this dye is not available, nor is it known whether the dye is a chloride or an acetate. The important point in its preparation is that it is made by dissolving 0.05 per cent of the dry dye in a solution having a pH between 2 and 3. In fact, this is the essential feature of the entire procedure. Whether this pH is obtained by the use of 0.05 N hydrochloric or 0.1 N acetic acid or a buffered solution is not important. This observation indicates that it is immaterial whether the basic fuchsin is a chloride or an acetate. Several samples of basic fuchsin of unknown origin have given almost equally good results. Safranin O Grubler has also been employed successfully at pH 2.5, but cleaner histological detail is obtained by the use of basic fuchsin. The most suitable concentration seems to be 0.05 per cent.

The use of a staining rack and dropping bottles rather than staining jars is recommended, since it permits the use of uncontaminated reagents.

Preliminary fixation of tissue can be accomplished by the common fixatives; formaldehyde solution U.S.P. (1:10), Zenker's fluid, Zenker's fluid plus dilute solution of formaldehyde or Bouin's fixative can be used with success. Better histological detail seems to be obtained by the use of Zenker's fixative without acetic acid.

When viewed under the low power of the microscope, the tissue is lightly stained. Gram-positive bacteria are deep violet and easily

identified with the low-power lens. Where cellular exudation is not too abundant, the deep red gram-negative bacteria can also be seen under the low-power lens in contrast to the lighter red nuclei. Under oil immersion both types are sharply differentiated.

Nuclear chromatin stains red; cytoplasm, faint yellow. The nuclei of nerve cells show differentiation of nucleoli and Nissl bodies. Axones are unstained. Myelin sheaths are violet. Fibrin and the fibers of connective tissue are pale pink. Erythrocytes are yellow.

Gram-positive bacteria are deep violet. Staphylococci, streptococci, mycelia of actinomycetes, and vegetative forms of spore-bearing species are solidly stained. Diphtheria bacilli show barred and granular forms. Human tubercle bacilli are less deeply stained than other gram-positive organisms and show distinct beading.

Gram-negative bacteria are uniformly red. The clubs of actinomycetes are also red and contrast sharply with the gram-positive mycelium.

The chief advantage of this stain is that it not only provides simultaneously good histological and bacterial differentiation but also stains all bacteria that may be present. This is particularly useful in the study of tuberculous tissue where very young tubercle bacilli may not be acid-fast, and secondary invaders are easily identified.

A New Method to Demonstrate Gram-Negative Organisms in Tissue Sections

(Krajian)

1. Prepare thin frozen or paraffin sections (5 to 7 microns) in usual manner, and bring them down to water.
2. Mordant in a 5 per cent aqueous solution of thorium nitrate for 5 minutes.
3. Rinse in tap water.
4. Stain in the following solution for 3 minutes:

Hematoxylin crystals -----	3 Gm.
Ferric ammonium sulfate -----	3 Gm.
Ferric chloride -----	3 Gm.
Potassium iodide -----	3 Gm.
Alcohol, 95 per cent -----	25 c.c.
Distilled water -----	25 c.c.

Dissolve the hematoxylin crystals in alcohol and the other reagents in water, and mix the two solutions together.

5. Wash in tap water.

6. Decolorize in 1 per cent acid alcohol until the excess color ceases to come off.

7. Wash in tap water.

8. Treat with equal volume of 10 per cent potassium iodide and 10 per cent zinc sulfate for 5 minutes.

9. Rinse in tap water.

10. Counterstain with carbol-fuchsin (Ziehl-Neelsen) for 7 to 10 minutes.

11. Wash in tap water.

12. Treat with a 5 per cent solution of sodium sulfite for 2 minutes.

13. Rinse in water.

14. Treat with a 3 per cent acetic acid solution for 3 minutes.

15. Blot, and apply aniline xylol (equal volume of aniline oil and xylol) for 30 seconds.

16. Treat with pure creosote from 2 to 5 minutes, agitating the section for even destaining and clearing. Stop when no more pink color is seen.

17. Clear 2 minutes in pure xylol and mount in gum dammar.

Gram-positive organisms assume hematoxylin color; gram-negative organisms, bright red.*

Thorium nitrate solution should be prepared fresh.

New and Rapid Staining Method for Gram-Positive and Gram-Negative Organisms in Frozen and Paraffin Sections*

Christian Gram in 1884 devised his method for the demonstration of bacteria in tissue sections. Weigert's modification of Gram's method has been useful for the demonstration of fibrin and bacteria and is still widely used, but both methods demonstrate only gram-positive organisms.

Since then, numerous attempts have been made to demonstrate both gram-positive and gram-negative organisms in paraffin sections of tissues that had been fixed in Zenker's solution or in formaldehyde solution, and methods accomplishing this purpose

*J. Lab. & Clin. Med., October, 1943.

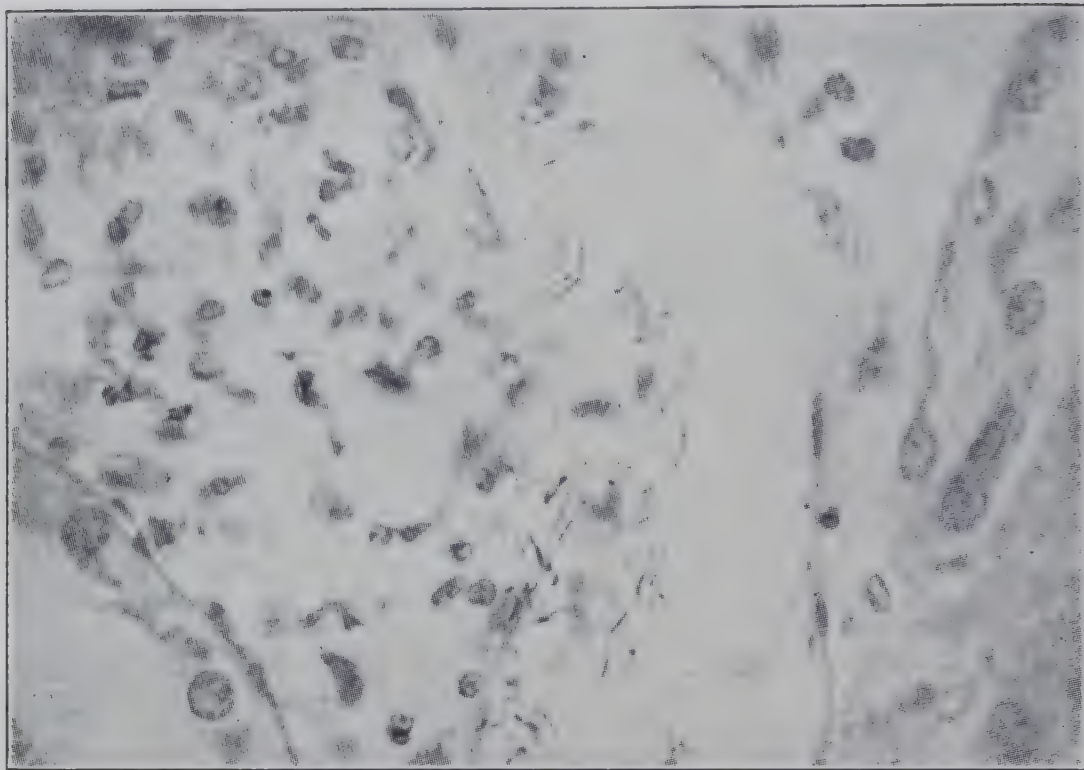


Fig. 99.—Streptococcus in kidney, stained blue, $\times 1620$.

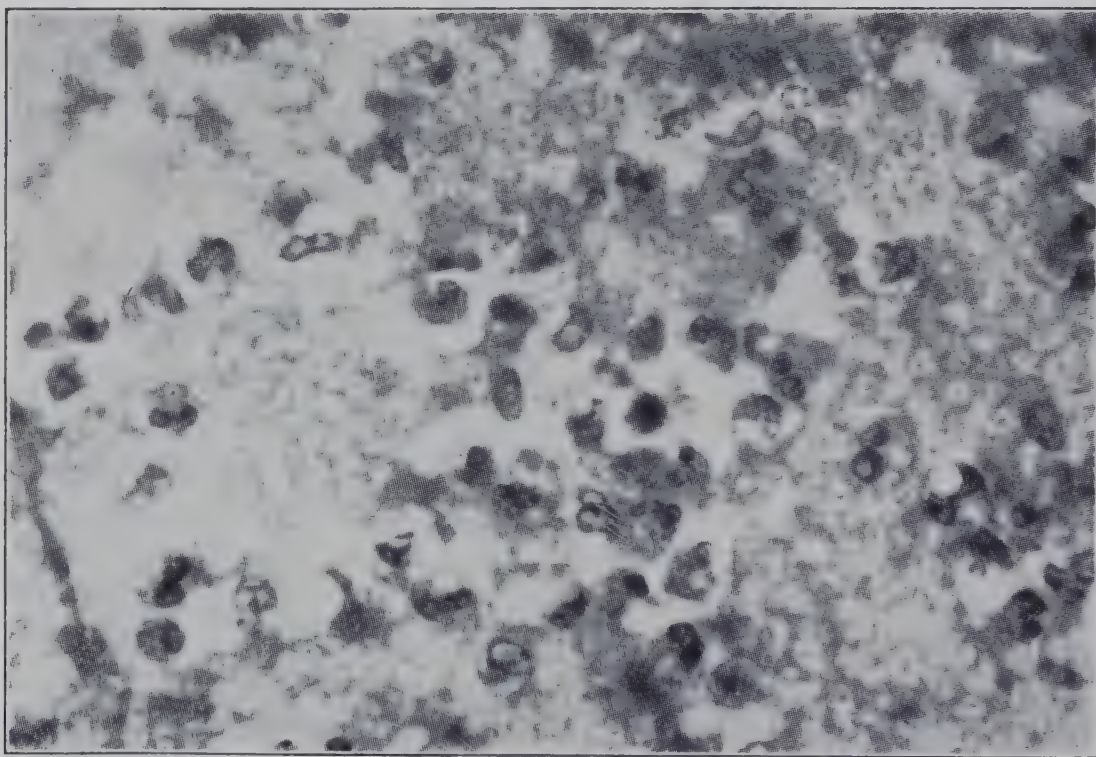


Fig. 100.—Friedländer's bacilli in pneumonia, stained red, $\times 1620$.

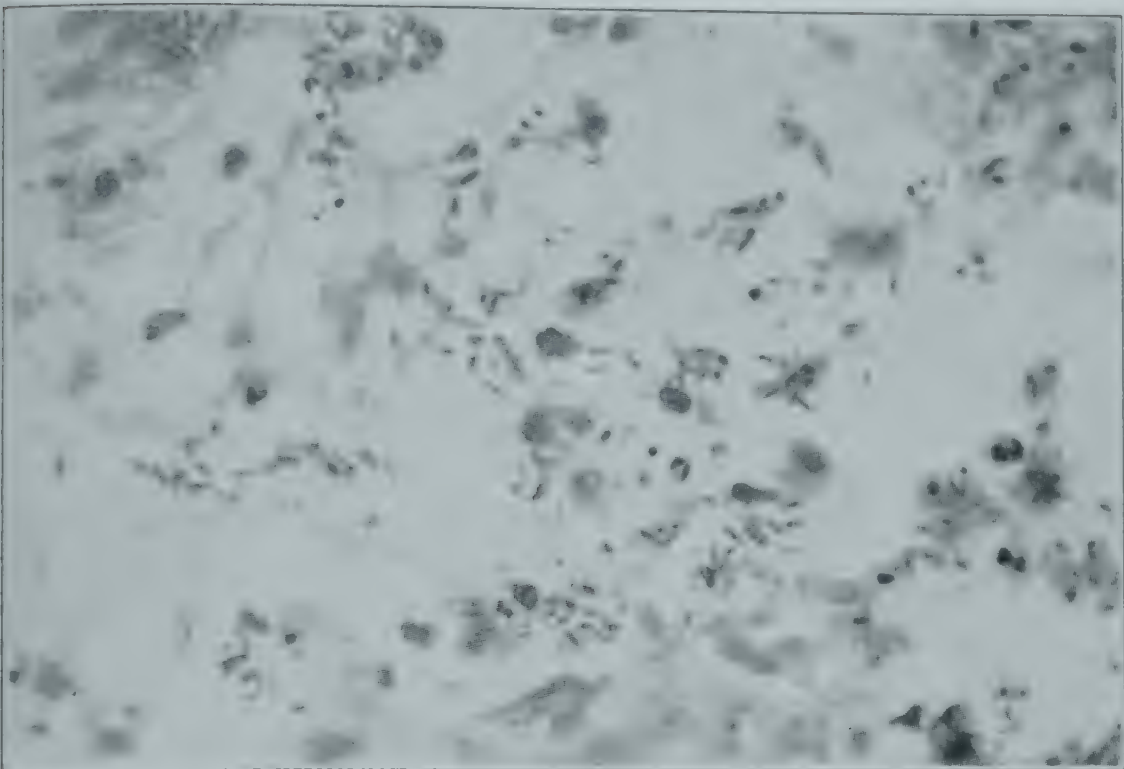


Fig. 101.—Sporotrichum in testicle, stained blue, $\times 1620$.

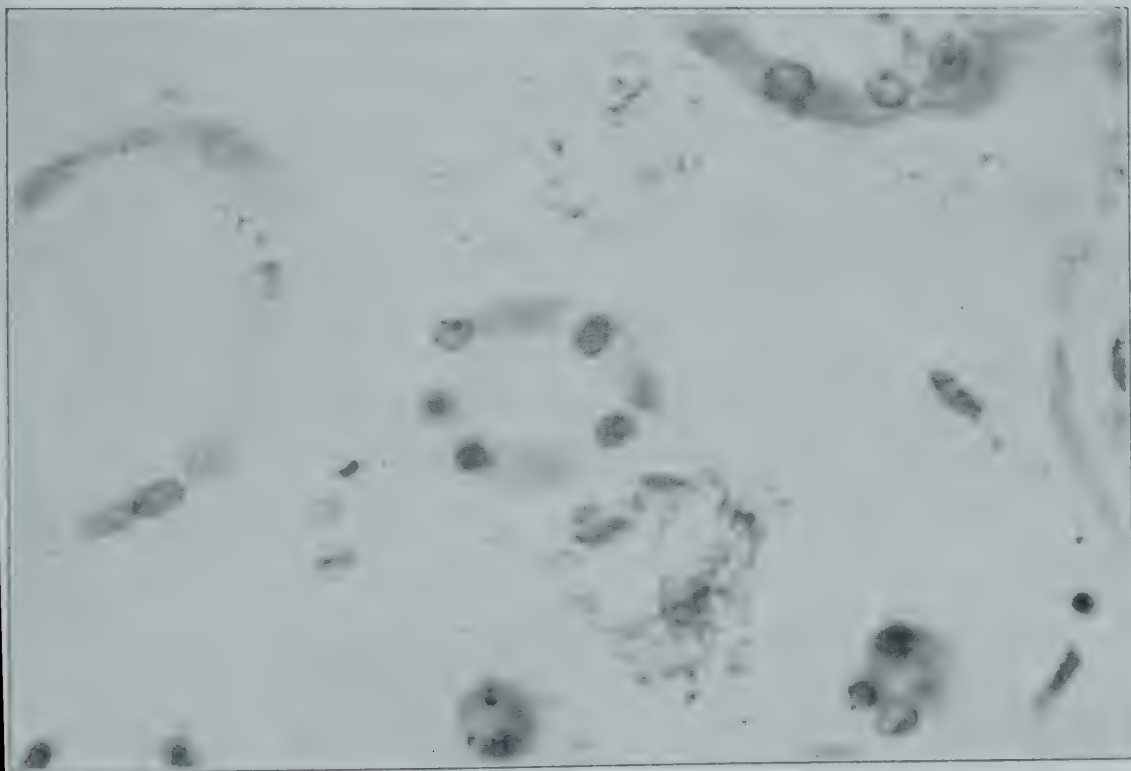


Fig. 102.—Colon bacilli† in kidney tubules, stained red, $\times 1620$.

have been reported by MacCallum, Lyon, Lillie, Brown and Brenn, Rudnikoff and Stawsky, Glynn, and others.

In these modifications, the staining solutions as devised by Gram remained practically unchanged. No attempt has been made to devise a new method by the use of some other dye or chemical, and no recommendations have been made as to the applicability to frozen sections.

The solutions used by Gram and used in the modifications of his method are: gentian violet, methyl violet, and crystal violet, as gram-positive stains; Gram's solution and compound solution of iodine, U.S.P. (Lugol's solution), as differentiators; rosaniline hydrochloride, basic fuchsin, dilute carbolfuchsin, and safranine as gram-negative stains; and acetone, alcohol, ether, and aniline oil-xylene as decolorizers.

The need for rapid diagnosis has led to the investigation of the usefulness of a different dye and different solutions. A new and simple method was devised which is easily applied to frozen and paraffin sections of formaldehyde-fixed tissue and which gives satisfactory simultaneous differential staining of gram-positive and gram-negative organisms. For example, four different sections (frozen or paraffin) mounted on the same slide, one of each of a kidney containing monilia, a lung with Friedländer's bacilli, a testicle containing sporotrichum, and a kidney with colon bacilli, were stained, with successful differentiation of the organisms by the method described. The technic has also been used on tissues containing streptococci, Welch bacilli, influenza bacilli, actinomyces, meningococci, gonococci, diphtheria bacilli, and Negri bodies. Similar results are obtained in another method recently described, but the time and material required have been greatly decreased in the present technic.

In this new method, the solutions used are: methylene blue as a gram-positive stain, fuchsin-creosote as a gram-negative stain, creosote-xylene as a differentiator.

Frozen Section Method

1. Prepare mounted frozen sections at 7 to 10 microns in the usual manner.
2. Stain for 3 minutes with alkaline methylene blue (Loeffler's).
3. Wash in tap water and dehydrate rapidly with 3 applications of anhydrous isopropanol or absolute ethyl alcohol.

4. Differentiate rapidly with creosote-xylene (1 part creosote plus 2 parts xylene), agitating slide constantly for 5 to 10 seconds.
5. Pour off and apply creosote-fuchsin (2.5 c.c. of a 6 per cent alcoholic basic fuchsin added to 50 c.c. of creosote-xylene), agitating slide about 15 to 20 times, changing the solution once.
6. Blot and apply creosote-xylene 2 or 3 times, agitating the slide constantly for even decolorization or until most of the excess red color leaves the section.
7. Blot, clear for 2 minutes in xylene and mount in gum dammar.

For Paraffin Sections

Deparaffinize section with two applications of xylene and two applications of absolute alcohol or isopropanol; bring down to tap water and then proceed with the method described.

With the use of this method, nuclei are red; gram-positive organisms, blue; gram-negative organisms, red; devitalized gram-positive organisms, red; monilias and actinomyces, blue; Negri bodies, bright red with bluish chromatin bodies; and fibrin, sometimes blue and sometimes red.

All the staining solutions are stable.

Modified Silver Stain of Microorganisms in Tissues*

(Steiner Method)

1. Cut sections 7 to 8 microns thick from paraffin-embedded tissue blocks, previously well fixed in 10 per cent formalin.
2. Attach sections to clean slides with Albumin Glycerine (Hartman and Leddon Company, Harleco, Philadelphia). When not in use this material should be kept in the refrigerator.
3. Place slides in glass racks. Metal racks and other metal instruments cannot be used. Use glass rods for transfer of the racks and porcelain or glass spoons for taking weights. Dishes in which silver nitrate solutions were used should be cleaned with nitric acid and rinsed thoroughly with distilled water.

The method:

1. Deparaffinize sections in two changes of fresh xylol not previously used. Pass through two changes of absolute alcohol not previously used and transfer to the following mixture of alcoholic

*Steiner, Gabriel: Modified Silver Stain of Microorganisms in Tissues, *Am. J. Clin. Path.* May, 1950. Published by The Williams & Wilkins Co.

uranium nitrate and alcoholic gum mastic solution for 5 or 6 minutes: 2 Gm. uranium nitrate C.P., 100 ml. absolute alcohol, 100 ml. 2 per cent alcoholic gum mastic. (Use 5 per cent stock solution of gum mastic with absolute alcohol in proportion of 40:60.)

The 5 per cent alcoholic gum mastic solution (absolute alcohol) can be kept permanently. Five grams powdered gum mastic (gum mastic powder, N.F., S. B. Penick and Company, New York), is dissolved in 100 ml. absolute alcohol. The solution is stirred several times with a thick clean glass rod. After standing for 24 to 40 hours it is carefully filtered several times through double or triple layers of filter paper until the solution is free of particles and entirely clear.

2. Wash rack with slides four or five times in distilled water until the milky appearance of the fluid disappears.

3. Impregnate in 0.1 per cent aqueous (distilled water) silver nitrate C.P. (which should be made freshly each time from a 1 per cent silver nitrate solution kept in an amber bottle in the dark) at 60 C. in the oven for 14 to 16 hours.

4. Wash four times in distilled water.

5. Dehydrate in graded alcohols, transfer from the absolute alcohol immediately to 2 per cent alcoholic gum mastic solution (not previously used) for 5 minutes.

6. Move rack with slides directly into 5 per cent warm catechol C.P. (Eastman Kodak Company) aqueous (distilled water), reducing solution for 1 to 1½ hours in warming oven (60° to 62° C.). Catechol C.P. is identical with pyrocatechin or orthodioxylene. First place the dish with catechol solution, without slides, in warming oven and put the slides into it after 10 minutes.

7. Wash thoroughly in distilled water.

8. Dehydrate in alcohol, clear in xylol, mount with clarite.

Prepare the uranium nitrate and the reducing solution freshly each time before use. Discard solutions after they have once been used. Once silver-impregnated sections have been put through the dehydrating series, the series should not be used again for the same process. It can be used, however, in routine hematoxylin-eosin and other aniline-dye staining processes.

The method is easy to handle and gives uniform results. The tissues stain yellowish, axis cylinders and reticulin fibers and fibrils are not stained, and microorganisms appear black in the light yellow background of the tissue and are easily detected. The

method has been applied to nervous tissue, and lung, liver, kidney and other tissues. Bacteria, spirochetes and fungi have been stained by this method.

ACID-FAST STAINS

Ziehl-Neelsen Method

The Ziehl-Neelsen method is the standard method of staining acid-fast bacilli in sections. It is used to stain both tubercle and leprosy bacilli.

1. Fix tissues in mercuric chloride or formaldehyde and make paraffin or frozen sections.
2. Remove paraffin with 2 changes of xylol and absolute alcohol.
3. Stain lightly in Harris' hematoxylin.
4. Wash in tap water until blue.
5. Stain in carbolfuchsin over a Bunsen burner for 10 minutes, gently steaming. This method is used for rapid diagnosis; better results are obtained if sections are left in carbolfuchsin solution 16 to 24 hours at room temperature.
6. Wash in water.
7. Decolorize for a few seconds in acid alcohol (1 c.c. hydrochloric acid in 99 c.c. 70 per cent alcohol).
8. Continue the decolorization in 70 per cent pure alcohol until no more pink color is seen.
9. Dehydrate in absolute alcohol, then blot.
10. Clear in 2 changes of xylol.
11. Mount in gum dammar.

Do not use carbolxylol for clearing, because it fades the red color of the bacilli.

Stain leprosy bacilli only 5 minutes by the steaming method, and decolorize 15 seconds in acid alcohol.

Leprosy and tubercle bacilli appear bright red, and nuclei of the cells take the hematoxylin stain.

New and Dependable Method for the Demonstration of Acid-Fast Organisms in Tissue Sections*

The importance of a consistently satisfactory and dependable method for the identification of tubercle and leprosy bacilli and of allied acid-fast organisms in the granulomatous lesions of biopsy and necropsy material cannot be over emphasized.

*Krajian, A. A.: Am. J. Clin. Path. 13: No. 5, 1943.

The methods described in microtechnics call for the application of a nuclear stain first, then the carbol fuchsin (Ziehl-Neelsen) for the staining of organisms, a decolorizer, and finally dehydrating and clearing.

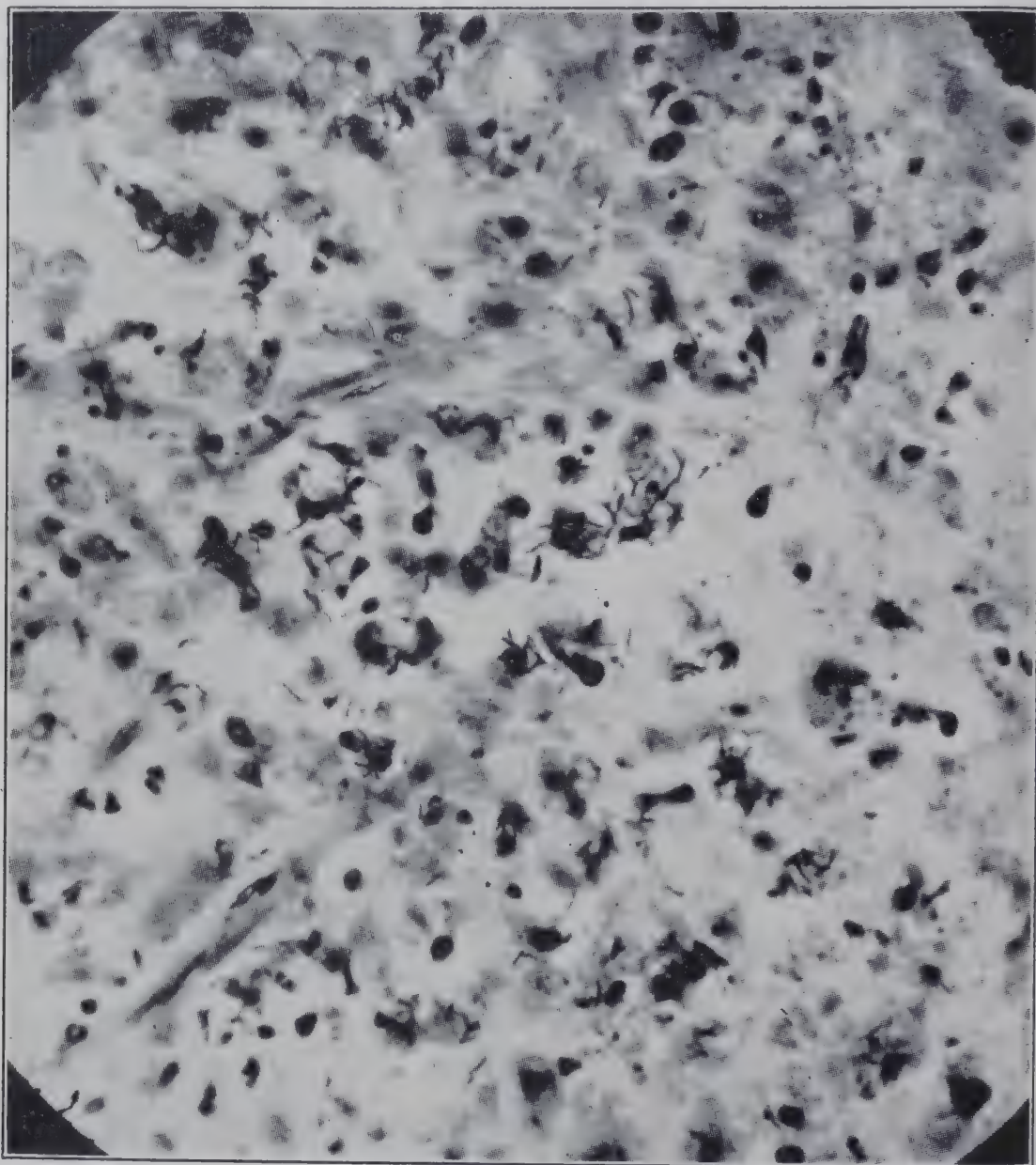


Fig. 103.—Tubercle bacilli in lung tissue, from a case of miliary tuberculosis, $\times 1620$. (Am. J. Clin. Path., May, 1943.)

The unsatisfactory feature in these accepted standard methods has always been in the use of the decolorizer. Carbol fuchsin stains not only the organisms, but it also is absorbed by the connective tissue system. If the decolorizer (acid alcohol) is overapplied

to remove the color from the connective tissue fibers, it will also affect the color of the organism and fade the nuclear stain. To overcome this obstacle, there has been developed a satisfactory and

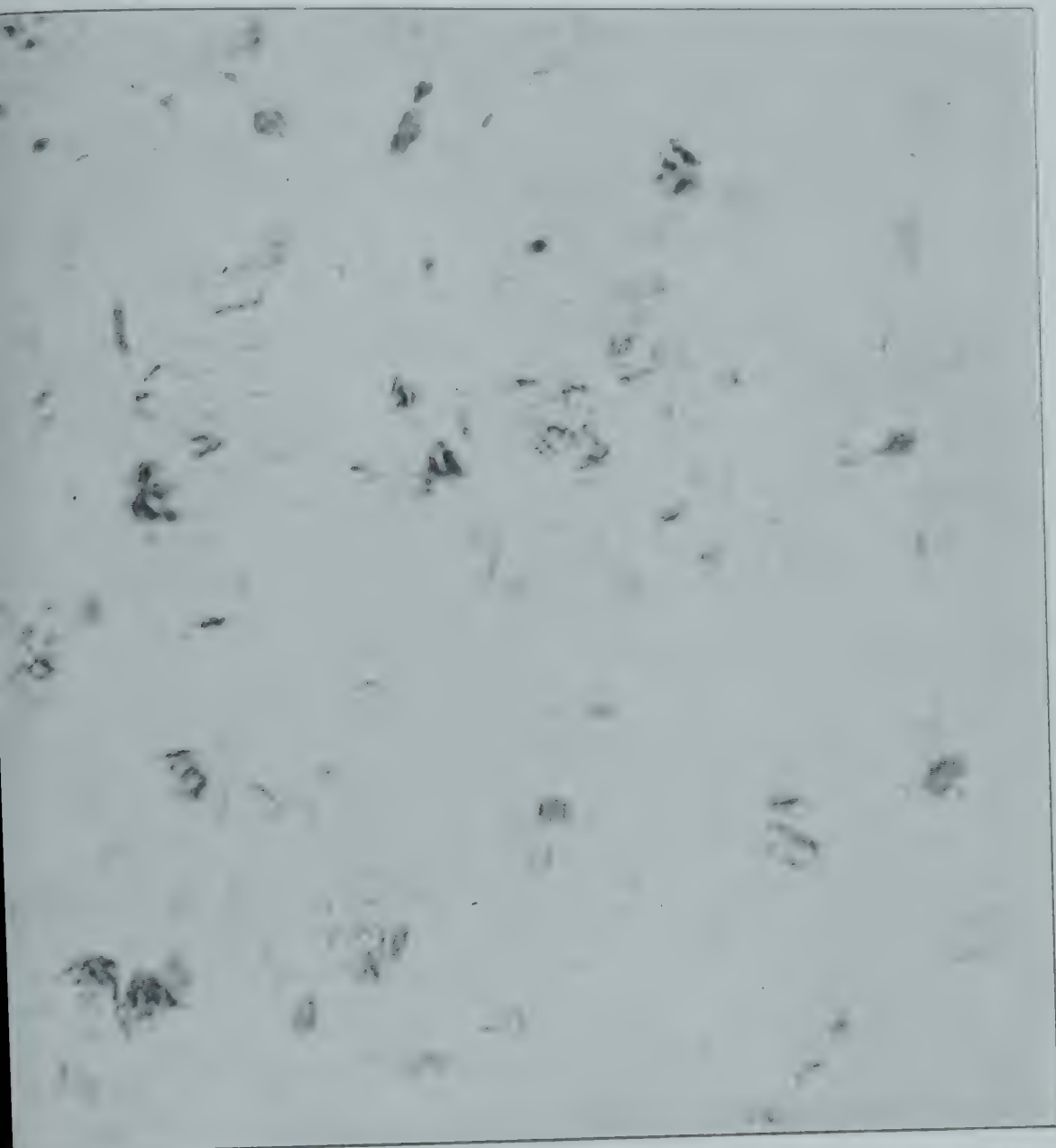


Fig. 104.—Leprosy bacilli in fibrous tissue, from a case of leprosy of arm, $\times 1620$.
(Am. J. Clin. Path., May, 1943.)

dependable method, making changes in the type of nuclear stain and decolorizer, and introducing a differentiating step. The method is easily applied to frozen and paraffin sections fixed in ordinary fixatives.

The method:

1. Cut frozen sections 7 to 10 microns.
2. Transfer to glass slide, dehydrate, blot in filter paper, and dip once in celloidin; blow over the surface until dry. Wash in tap water until surface is wet.
3. Place on a tripod or on warming table, apply carbol fuchsin, and steam gently with a flame for 3 minutes.
4. Discard the staining solution, wash with tap water, and apply arsenic acid alcohol (1 Gm. of arsenic acid in 100 c.c. of 60 per cent ethyl alcohol) with a medicine dropper 2 to 4 times, agitating the slide constantly for even destaining, or until most of the red color is removed.
5. Wash in tap water and apply Loeffler's methylene blue solution for 2 minutes.
6. Wash in tap water and dehydrate with 3 applications of anhydrous isopropanol or absolute ethyl alcohol.
7. Immediately apply iso-creosote (equal parts of anhydrous isopropanol or absolute ethyl alcohol and beechwood creosote) 2 or 3 applications, agitating the slide constantly for even differentiation. This removes the excess blue stain from tissue as well as from organisms. This is the crucial step; if overdifferentiated, the background will be very light blue without affecting the organisms; light blue is the right color.
8. Blot immediately in filter paper.
9. Clear in 3 changes of xylene, 1 minute each.
10. Mount in gum dammar.

For paraffin sections, remove paraffin with two applications of xylene, 2 minutes each, remove xylene with 2 applications of isopropanol or absolute alcohol, wash in tap water, and proceed with method described.

Figs. 103 and 104 demonstrate the results obtained with this method.

Acid-Fast Stain on Paraffin Sections

(Fuller)

This method is excellent for photomicrographic purposes, as tubercle bacilli are stained brilliantly red while nuclei take various shades of green, so that good contrast is obtainable.

The method:

1. Deparaffinize sections in usual manner and run slides down to water.

2. Mordant in a 5 per cent iron alum solution in the paraffin oven for 5 minutes at 45° to 50° C.

3. Rinse in water.

4. Stain in hematoxylin in paraffin oven for 5 minutes. (Dissolve 1 Gm. of certified hematoxylin in 80 c.c. of hot distilled water, cool and add 10 c.c. of glycerin and 10 c.c. of 95 per cent alcohol.)

5. Place directly into the picric acid solution for 5 minutes or longer if necessary, until the stain remains only in nuclei (picric acid solution is prepared by adding 2 parts of saturated alcoholic solution of picric acid to 1 part of 95 per cent alcohol).

6. Wash thoroughly under running water, 15 minutes or longer, or until sections are free of picric acid.

7. Place section on heating bar or warming table and pour fuchsin solution over them. Heat until steam comes off, but do not boil. (Fuchsin solution is prepared by mixing 16 c.c. of saturated alcoholic solution of basic fuchsin with 84 c.c. of aniline water.)

8. Decolorize in acid alcohol for a few seconds, until the fuchsin starts coming off in clouds; rinse in tap water and continue decolorization until the sections are barely pink. (Acid alcohol is prepared by mixing 3 c.c. concentrated hydrochloric acid with 100 c.c. of 95 per cent alcohol.)

9. Rinse in about 1 per cent ammoniated water for a few seconds.

10. Wash thoroughly under running water for 10 minutes.

11. Stain in light green solution for 5 minutes. (It is a 1 per cent solution of light green.)

12. Wash in water, and dehydrate rapidly in 95 per cent alcohol, followed with absolute alcohol.

13. Clear in xylol for 5 minutes and mount in gum dammar.

Modification of Ziehl-Neelsen Method

(Krajian)

1. Stain thin frozen, paraffin, or celloidin sections lightly in alum hematoxylin (Harris).

2. Wash in tap water until blue.

3. Stain for 5 minutes with carbolfuchsin, gently steaming. (Keep slide fully covered with staining solution.)

4. Wash in tap water.

5. Destain with picric acid alcohol (1 part of saturated aqueous solution of picric acid and 1 part of 95 per cent alcohol) for 15 to 30 seconds, changing the solution 2 or 3 times and agitating the section for even destaining.

6. Wash thoroughly in large basin of tap water.
7. Treat with 2 per cent solution of sodium cyanide for 10 to 15 seconds, and wash thoroughly in tap water.
8. Dehydrate completely with several changes of absolute alcohol or anhydrous isopropanol (this also removes the excess fuchsin stain).
9. Blot dry and clear in 2 changes of pure xylol (2 minutes each).
10. Mount in gum dammar.

Do not use carbolxylol for clearing, because it fades the red color of the bacilli.

By this improved technic the tubercle and leprosy bacilli appear bright red, and the nuclei blue.

Flexner's Method of Staining Leprosy Bacilli in Tissue Sections

1. Stain sections in alum hematoxylin (Harris) to obtain a sharp nuclear stain.
 2. Wash in water.
 3. Stain in Ziehl-Neelsen's carbolfuchsin for 2 to 5 minutes, steaming, or 30 to 60 minutes at room temperature.
 4. Wash in water.
 5. Treat with Gram's iodine solution for $\frac{1}{2}$ to 1 minute.
 6. Wash in water.
 7. Blot in filter paper, clear, and differentiate in aniline oil.
 8. Clear in pure xylol. Mount in gum dammar.
- Leprosy bacilli stain red; nuclei, light blue.

Giemsa Stain

For Bone Marrow Study

Fix very thin pieces of bone marrow in Zenker's fluid for 12 to 16 hours. Wash under running water for 16 to 24 hours. Embed in paraffin in usual manner and cut sections 5 microns or thinner.

1. Deparaffinize with 2 applications of xylol, 2 minutes each.
2. Remove xylol by 2 applications of absolute alcohol.
3. Treat with tincture of iodine for 5 minutes to remove the bichloride precipitate.
4. Treat with 1 per cent aqueous solution of sodium thiosulfate for 5 to 10 minutes.
5. Wash thoroughly in tap water followed with neutral distilled water.

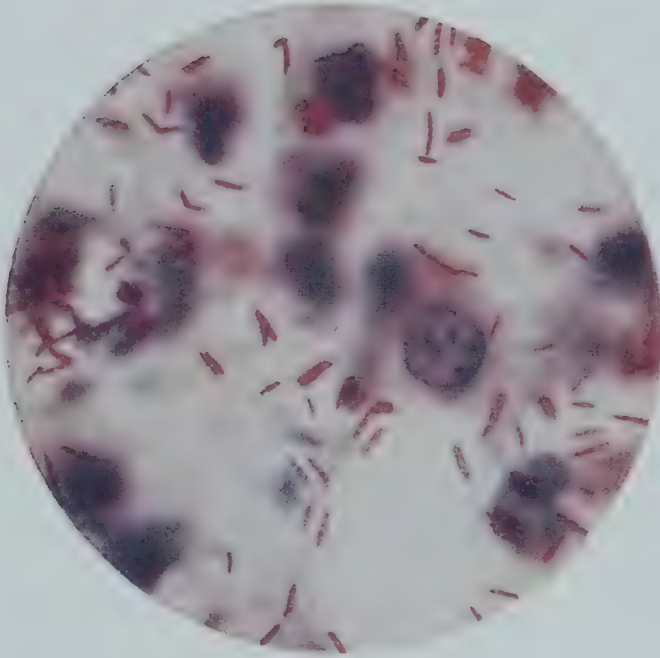


PLATE VI

Section from biopsy of arm stained by modified Ziehl-Neelsen method (Kra-jian). Leprosy bacilli, bright red; nuclei, reddish blue. Frozen section.

Magnification 1,450 diameters. Original on Kodachrome. Made with Bausch & Lomb 2.5 MM apochromatic objective of 1.30 N.A. and 12.5X compensating eyepiece.

6. The composition of Giemsa's stock solution:

Azure II eosin -----	3	Gm.
Azure II -----	0.8	Gm.
Alcohol, methyl (Merck) -----	375	c.c.
Glycerin, C.P. -----	125	c.c.

Mix above reagents and filter.

7. For staining sections prepare the following solution:

Giemsa's stock solution -----	3	c.c.
Methyl alcohol (Merck) -----	3	c.c.
Neutral distilled water (add 2 drops of 1 per cent sodium bicarbonate to 100 c.c. distilled water) -----	100	c.c.

8. Place sections in the above dilute Giemsa solution. Change the stain twice and leave in the third solution overnight.

9. Place sections in distilled water.

10. Differentiate each slide separately in 95 per cent alcohol to which a few drops of colophonium in absolute alcohol are added.

11. Dehydrate with 2 changes of absolute alcohol.

12. Clear in neutral xylol and mount in cedarwood oil or gum dammar.

Nuclei of cells and neutrophile granules stain red violet; basophile granules, blue violet; cytoplasm of lymphocytes, blue; azure granules and red blood cells, pale red.

Giemsa Stain

For Protozoological and Hematological Work

For the demonstration of protozoa and bacteria, thin pieces of tissue are fixed in mercuric alcohol (4 Gm. bichloride of mercury in 95 per cent alcohol) for 2 days, changing the fixing solution after 24 hours.

For hematological work thin pieces of tissue are fixed in Helly's fluid (Zenker formol) for 12 to 16 hours, and washed under running water for 24 hours.

After primary fixation they are dehydrated and embedded in paraffin in usual manner.

1. Cut sections 3 to 5 microns.

2. Deparaffinize with 2 applications of xylol, 2 minutes each.

3. Remove xylol by 2 applications of absolute alcohol or anhydrous isopropanol.

4. Treat with tincture of iodine for 5 minutes to remove the bichloride precipitate.

5. Treat with 1 per cent aqueous solution of sodium thiosulfate for 5 to 10 minutes.

6. Wash thoroughly in tap water followed with neutral distilled water.

7. Stain in a freshly prepared dilute Giemsa stain for 16 to 24 hours. The dilution is prepared by adding 1 drop of stock Giemsa stain to 1 c.c. of distilled water. After the first hour the dilute Giemsa mixture is replaced by a fresh supply:

8. Wash in distilled water.

9. Dehydrate and differentiate in:

Acetone, 9 parts plus 1 part xylol
 Acetone, 5 parts plus 5 parts xylol
 Acetone, 1 part plus 9 parts xylol

10. Clear in neutral xylol. Mount in gum dammar.

Sometimes better results are obtained by clearing sections in carbolxylol or creosote first, followed with neutral xylol.

The nuclei of protozoa stain red; nuclei of cells and neutrophile granules, red violet; basophile granules, blue violet; cytoplasm of lymphocytes, blue; azure granules and red blood cells, pale red.

Giemsa Stain on Tissue Sections

(Schilling method, modified by Gradwohl)

Susa fixative:

Mercuric chloride -----	4.5 Gm.
Sodium chloride -----	0.5 Gm.
Trichloroacetic acid -----	2.0 Gm.
Glacial acetic acid -----	4 c.c.
Formaldehyde -----	20 c.c.
Distilled water -----	80 c.c.

Fixation, dehydration, and embedding:

1. Immerse the tissue in Susa fixative for 5 to 48 hours, depending on the size.

2. Wash in tap water 3 to 5 hours, changing the water frequently.

3. Place in 50 per cent alcohol for half a day, changing alcohol several times.

4. Transfer to 70 per cent alcohol for half a day, changing alcohol several times.

5. Transfer to 95 per cent alcohol for half a day, changing alcohol several times.
6. Place in absolute alcohol for half a day, changing alcohol several times.
7. Place in absolute alcohol not longer than 1 hour. Larger blocks should be left longer than 1 hour.
8. Clear in pure xylol. The tissue must be transparent.
9. Place the tissue in a mixture of equal parts of paraffin and xylol at a temperature not to exceed 37 to 40° C. overnight. Use special embedding paraffin with a melting point of 56 to 58° C. Tissuemat may be used.
10. Keep the paraffin oven at a temperature 2 degrees above melting point of paraffin.
11. Place tissues in pure paraffin or Tissuemat in paraffin oven 4 hours. Transfer to fresh paraffin or Tissuemat in paraffin oven 2 to 4 hours. Embed in fresh paraffin or Tissuemat in usual manner.

Staining:

1. Cut sections about 5 microns and mount on slides using Mayer's egg albumen fixative and allow to dry thoroughly.
2. All solutions for staining sections should be in staining jars.
3. Place the slides in xylol for 5 minutes to remove the paraffin.
4. Carry through.

Absolute alcohol, 1 minute

Alcohol, 95 per cent, 1 minute

Alcohol, 80 per cent, 1 minute

Alcohol, 70 per cent, 1 minute

5. Place iodine alcohol (100 c.c. 70 per cent alcohol and 1 c.c. tincture of iodine) for 1 to 2 hours, or until tissues become of brown color. Omit this step for formaldehyde-fixed sections.
6. Treat briefly in 0.2 per cent sodium thiosulfate solution not longer than 10 to 15 minutes or until the brown color is removed.
7. Wash 30 minutes in a jar of tap water.
8. Wash 30 minutes in a jar of distilled water.
9. Dilute Giemsa stain by adding 1 drop of Giemsa stain to each cubic centimeter of neutral distilled water, shaking briefly after the addition of each drop. Avoid shaking too vigorously.
10. Stain for 30 minutes on a staining bridge, flooding the slide with Giemsa stain.
11. Renew the stain at the end of 30-minute staining period, diluting a fresh supply for the restaining process. Restain 30 minutes.

12. Wash briefly in a jar of distilled water.

13. Place the slide under the low-power objective of a microscope and decolorize as follows:

Use a weak solution of acetic acid for decolorization. This is made by adding a few drops of 1 per cent acetic acid to 50 c.c. distilled water. Have on hand a jar of slightly alkaline water, made by adding 2 or 3 drops of 1 per cent potassium carbonate solution to 100 c.c. tap water.

14. Place the Giemsa-stained slide under microscope, low-power magnification.

15. Flood the slide with the weak acetic acid solution, and as soon as the pink color of the eosin is observed, place the slide in the jar containing slightly alkalized water. The rapidity of this change is very important, because the section will continue to be decolorized as long as the acid remains not neutralized on tissue.

16. Dehydration is carried out in staining jars. The solutions are as follows:

1. Xylol -----	5 parts
Acetone -----	95 parts
2 and 3	
Xylol -----	30 parts
Acetone -----	70 parts

17. Place the slide in solution No. 1 for about 5 to 10 seconds.
 in solution No. 2 for about 5 to 10 seconds.
 in solution No. 3 for 10 seconds or more.

18. Place in xylol. If the section does not become transparent, place again for a few seconds in solution No. 3 then back in xylol. Continue this process until the section is clear, then leave in xylol for several minutes. Keep the acetone solutions tightly stoppered to prevent rapid evaporation and absorption of undue amount of moisture. Mount in gum dammar.

AMOEBA STAIN

Heidenhain's Iron Hematoxylin

Heidenhain's iron hematoxylin is a standard method to demonstrate the cysts of *Amoeba histolytica*, chromatin, nuclear membrane, centrosomes, and various secretory products. It is applicable after any standard method of fixation. Zenker's fluid and alcohol are preferable.

1. Cut very thin, frozen, paraffin, or celloidin sections. Remove the precipitate from bichloride fixed sections by treatment with iodine, and place them for 1 to 24 hours in a staining dish containing 5 per cent aqueous iron alum (iron and ammonia alum, 5 Gm., dissolved in 100 c.c. distilled water).

2. Wash in tap water.

3. Place in staining dish containing 0.5 per cent solution of hematoxylin for 1 to 4 hours. It is prepared as follows:

Hematoxylin crystals -----	0.5 Gm.
Alcohol, 95 per cent -----	10 c.c.
Distilled water -----	100 c.c.

First dissolve hematoxylin in alcohol, then mix with distilled water. It takes several days for the solution to ripen. It keeps well in a stoppered bottle.

4. Wash in tap water.

5. Differentiate in a 2 per cent aqueous solution of iron alum. Control the destaining under the microscope by taking the section out of iron alum solution, dipping it in water, and examining under low-power magnification. Repeat the process until sections are suitably destained.

6. Wash in a large basin of tap water for several minutes until all traces of iron alum are removed from the section.

7. Dehydrate first in 95 per cent alcohol and then in absolute alcohol.

8. Blot and clear in 2 changes of xylol.

9. Mount in gum dammar. The nuclei of cysts, amoeba, chromatin, and nuclear membrane stain blue. This is a very valuable stain.

Krajian's Elastic Tissue Stain

For the Demonstration of Amoeba in Tissue Sections

1. Prepare thin, frozen, paraffin, and celloidin sections in usual manner.

2. Stain in Krajian's elastic tissue solution (see page 165) for 5 minutes.

3. Wash in tap water.

4. Destain in 1 per cent acid alcohol until the background begins to clear.

5. Wash in tap water.

6. Treat with a 5 per cent aqueous solution of zinc sulfate for 2 minutes.

7. Rinse in tap water.

8. Counterstain for 2 minutes in van Gieson solution.

9. Rinse in tap water and blot dry.

10. Differentiate and dehydrate with oil origanum-absolute alcohol (equal parts) solution for 2 minutes.

11. Clear in carbolxylol for 2 minutes; follow with pure xylol for 3 minutes.

12. Mount in gum dammar.

The nucleoli of amoeba stain a brownish-blue color; cytoplasm, pink; connective tissue, dark red.

A rapid and colorful method.

NEGRI BODY STAIN

To establish the diagnosis of rabies or hydrophobia, it is necessary to find in the nerve cells of the central nervous system the peculiar bodies known as "Negri bodies." These are generally round or oval and stain deeply with fuchsin; their granules take a blue color.

Goodpasture's Carbolfuchsin and Methylene Blue Method

1. Fix suspected brain tissue in Zenker's fluid, wash, dehydrate, and embed in paraffin in the usual manner.

2. Cut very thin sections.

3. Remove mercury precipitate by treating in tincture of iodine, then in 5 per cent sodium hyposulfite.

4. Wash very thoroughly in tap water.

5. Stain in the following stain from 10 to 30 minutes:

Alcohol, 20 per cent -----	100	c.c.
Phenol (pure) -----	1	c.c.
Aniline oil -----	1	Gm.
Basic fuchsin -----	0.5	Gm.

The finely powdered or granular dye is easily dissolved, and the solution is ready for use.

6. Wash off excess stain in running water.

7. Blot with fine filter paper.

8. Decolorize in 95 per cent alcohol until section becomes pink.

9. Wash off in tap water and counterstain for 15 to 60 seconds with Loeffler's methylene blue.
10. Wash in tap water.
11. Decolorize, and at the same time dehydrate, in absolute alcohol for a few seconds until the excess blue is removed.
12. Clear in 2 changes of pure xylol.
13. Mount in gum dammar.

This method is very useful for the demonstration of Negri bodies, bacteria, and rickettsia. The staining solution is not stable. It will be convenient to keep the required alcoholic solution of fuchsin on hand, adding it to the phenol and aniline oil at the time of use.

Negri Body Stain in Tissue Section

Zenker's fixed brain tissue is embedded in paraffin in the usual manner, and thin sections are cut and dried.

1. Remove paraffin with 2 applications of xylol, 2 minutes each.
2. Wash with 2 applications of anhydrous isopropanol or absolute alcohol, 1 minute each.
3. Treat with tincture of iodine for 10 minutes to remove the bichloride.
4. Wash in isopropanol or 95 per cent alcohol.
5. Wash thoroughly in water.
6. Stain in the following dilute stock solution for 7 to 10 minutes, gently steaming.

Stock solution:

Basic fuchsin -----	1	Gm.
Methylene blue -----	0.5	Gm.
Glycerin -----	50	c.c.
Alcohol, 95 per cent -----	50	c.c.

Dissolve fuchsin and methylene blue in alcohol, add glycerin and mix thoroughly.

Dilute solution: One part of the above stock solution mixed with 30 parts distilled water.

7. Rinse in tap water and blot.
8. Treat with anhydrous isopropanol or absolute alcohol for 15 to 25 seconds.
9. Blot dry and immediately dip in and out in carbolxylol until the background is completely red. Control this step under low

magnification of microscope; if the nuclei are a very deep blue, continue the treatment further with carbolxytol.

10. Clear in 2 changes of xylol, 2 minutes each.

11. Mount in gum dammar.

The stock staining solution is stable and keeps for a long period. Shake thoroughly before diluting it.

The diluted solution does not keep more than 15 minutes, and for this reason fresh solution should be prepared before use.

This is a highly satisfactory method to demonstrate Negri bodies in tissue sections.

Negri Bodies in Smear Preparation

(Frothingham)

Small pieces of gray matter are chosen from the hippocampus or vermis of cerebellum, and placed on a clean slide. Thin smears are prepared and air-dried. Then they are fixed in absolute alcohol for a few seconds.

Staining solution:

Saturated alcoholic solution of basic fuchsin ----	6 drops
Saturated alcoholic solution of methylene blue --	4 drops
Distilled water -----	30 c.c.

Stain smears in above solution (cold) for 10 to 20 seconds, blot dry, and examine under oil-immersion lens.

The Negri bodies are exceptionally brilliant red with blue granules.

SPIROCHETE STAINS

Among numerous similar organisms, *Spirochaeta pallida* stands out as peculiar in its resistance to stains. Many methods have been devised to demonstrate it, both in living and dead forms.

Methods are presented for the detection of *Spirochaeta pallida* by dark-field illumination, and by staining methods in smears and tissues.

Dark-Field Illumination of Living Spirochetes in Tissue Fluids

1. Moisten a swab in alcohol and roughly rub the lesion until it bleeds.

2. While waiting for the bleeding to stop, swab the lesion from time to time. After a few minutes a clear serous exudate, free from blood, will appear from the abraded surface.

3. Take up 2 drops of normal salt solution with a sterile platinum loop and place them on the center of a glass slide.

4. Take up 2 drops of the clear serous exudate from the lesion with the sterile platinum loop and mix with the saline drops on the slide.

5. Place a cover slip on this mixture and gently press it down with the handle of the platinum loop.

6. Examine it under the dark-ground microscope for spirochetes.

The examination should be systematic; every field should be examined with a mechanical stage, continuously focusing up and down with the fine adjustment of microscope to find the field rich with spirochetes. At least 4 or 5 slides should be carefully examined before pronouncing the case negative.

Staining *Spirochaeta Pallida* in Smears

Numerous methods for staining *Spirochaeta pallida* in smears have been devised and published, but these have not been found satisfactory in our hands. Only the following methods give good results.

Reliable Method for Staining *Spirochaeta Pallida* in Smears* (Krajian)

For the identification of *Spirochaeta pallida* in serous exudates from a suspected superficial lesion, the methods available include immediate examination of the material by dark-field procedure, the application of various stains to the smear, and the India ink method.

A staining method is here described which gives more dependable results than other methods, such as Giemsa's, Fontana's, Ghoregeb's, Gelarie's, and others with which we are acquainted.

Experience shows that, except in the hands of well-trained examiners, dark-field examinations are not dependable, and the proportion of positive findings is much below that actually possible. The biopsy method for demonstrating spirochetes is most satisfactory but entails a certain amount of inconvenience and added cost, which prevents the physician from performing the test on doubtful lesions of skin and mucous membrane. More frequent testing and the consequent finding of syphilis in a higher proportion of cases should

*Reprinted from the Arch. Dermat. & Syph. 38: 427, 1938.

result from the availability of a dependable method which can be used conveniently in a well-equipped laboratory. The silver stain for smears described here is capable of impregnating within a 15 minute period even the most resistant spirochetes and offers both convenient and permanent records.

It is, of course, admitted that recognition of the morphological characteristics of spirochetes on the surface of mucosal lesions may be of less significance than their recognition deep in the tissue of biopsy specimens, but the finding of spirochetes with the morphological characteristics of *Spirochaeta pallida* in smears from a suspicious lesion constitutes at least presumptive evidence of the syphilitic character of the lesion.

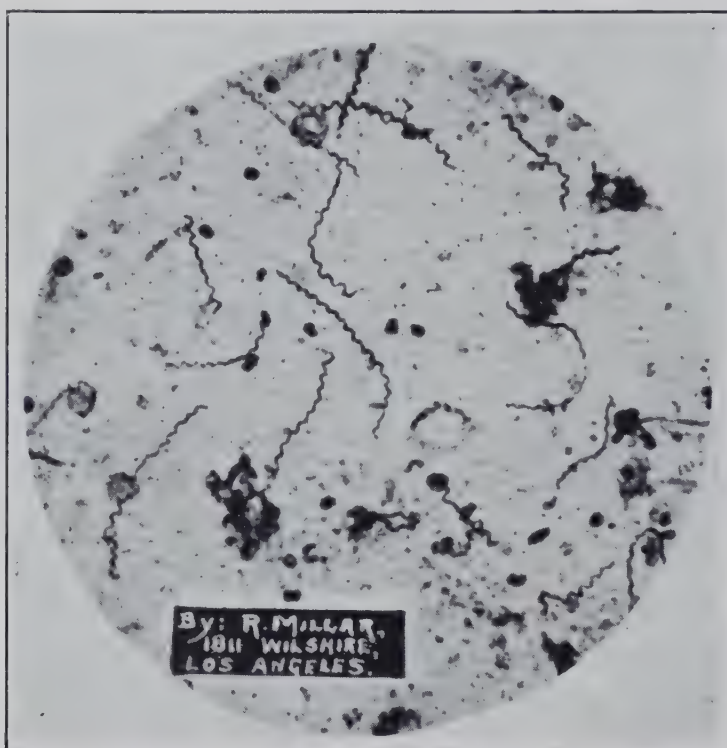


Fig. 105.—Smear from a cervical chancre stained by Krajan method.

Technic:

Moisten a swab in alcohol and roughly rub the lesion until it bleeds. Several minutes after all the bleeding stops, a clear serous exudate will appear on the abraded surface; from this fluid prepare and air-dry smears. Flood each smear for 5 minutes with a warm No. 1 solution (uranium nitrate, 1 Gm.; 85 per cent formic acid, 3 c.c.; glycerin C.P., 5 c.c.; acetone, 10 c.c.; and 95 per cent alcohol,

10 c.c.), wash in distilled water and treat with a weak solution of gum mastic (3 drops of saturated alcoholic solution of gum mastic mixed with 7 c.c. of 95 per cent alcohol) for 2 minutes. Pour off the gum and blow the breath over the surface of the smear; then rinse in distilled water. Set the slide on a metal stand or tripod, flood with a 1 per cent aqueous solution of silver nitrate, heat over a burner until bubbles begin to form (do not boil), and keep at that temperature for 3 minutes. Repeat the silvering process once, pour off the silver without washing, apply a thin coat of developing solution, and leave the slide under an electric light for 2 minutes, then warm gently with a flame. The developing solution consists of hydroquinone, 0.31 Gm.; sodium sulfite, 0.06 Gm.; solution of formaldehyde, 40 per cent neutral, 2.5 c.c.; pyridine, 2.5 c.c.; saturated solution of gum mastic in 95 per cent alcohol, 2.5 c.c., and distilled water, 15.0 c.c. Wash the slide with distilled water, dry on filter paper, and examine under an oil-immersion lens. For permanent mounts the preparation may be dehydrated with absolute alcohol, blotted, cleared in xylol, and mounted in gum dammar.

Stained smears should be brown.

No. 1 solution is stable and keeps for long periods.

Silver solution should be prepared from a 10 per cent stock solution each time.

Dilute gum mastic should be prepared fresh.

The developing solution keeps well for from 2 to 3 weeks in a light room, after which it deteriorates, the gum mastic separating and settling to the bottom of the container. When this occurs, a fresh supply should be prepared.

It is important to use fresh developing solution; otherwise the organisms will fail to stain.

Fontana-Hage Silver Method

1. Make thin smears from suspected material and dry in the air without warming.
2. Fix for 1 minute in Rouge's fluid.

Formaldehyde, commercial	-----	20 c.c.
Glacial acetic acid	-----	1 c.c.
Distilled water	-----	100 c.c.

3. Wash quickly in distilled water.

4. Flood the slide with the mordant:

Phenol crystals	1 Gm.
Tannic acid	5 Gm.
Distilled water	100 c.c.

Heat the slide over a flame until the fluid steams, remove from the flame, and let fluid remain on slide for 30 seconds.

5. Wash for 30 seconds under running water, rinse several times in distilled water.

6. Stain the slide in a 0.25 per cent solution of ammoniacal silver nitrate by warming the flooded slide over a flame until steam rises and place aside for 30 seconds. Immediately after flooding the slide with this solution, add 1 drop of strong ammonium hydrate to it. A brownish scum should appear on warming the slide.

7. Rinse in distilled water.

8. Blot.

Examine with oil-immersion objective. Spirochetes stain black; background stains yellow.

This method cannot be depended upon for constant good results.

Staining Methods for *Spirochaeta Pallida* in Tissues

(Levaditi and Manouelian Method)

1. Fix thin blocks of tissue in a 10 per cent formaldehyde solution 24 hours or longer.

2. Harden in 95 per cent alcohol for 24 hours.

3. Place the block in distilled water and wait until it sinks to the bottom of the container.

4. Impregnate in a 2 per cent aqueous silver nitrate solution for from 3 to 6 days in an incubator at 37° C.

5. Rinse the block in distilled water for 2 minutes.

6. Reduce for 24 hours at room temperature in the following solution. Leave the container in a dark place.

Pyrogallie acid	3 Gm.
Formaldehyde, commercial	5 c.c.
Distilled water	100 c.c.

7. Wash in distilled water for 15 minutes.

8. Dehydrate in graded alcohols, commencing with 70 per cent alcohol, finally with absolute, then clear and embed in paraffin in usual manner.

9. Cut thin paraffin sections, about 5 microns.

10. Dissolve the paraffin in xylol and mount in gum dammar. Spirochetes appear jet black with a yellow background.

The Levaditi method has been the standard for many years, but recently more rapid and improved methods have been devised which are far superior to Levaditi's.

Dieterle Stain for *Spirochaeta Pallida**

1. Fix tissues in 10 per cent formaldehyde and make frozen, paraffin, or celloidin sections in the usual manner. The paraffin or celloidin must be removed from the section as usual, with xylol for the former, acetone for the latter.

2. Place in pure pyridine for 15 minutes and wash thoroughly in distilled water.

3. Place in 1 per cent uranium nitrate in 70 per cent alcohol for 30 minutes at 55° C. The uranium solution may vary in strength with advantage, even up to 5 per cent.

4. Wash for a moment in distilled water.

5. Pass through 95 per cent alcohol.

6. Place individually in an absolute alcoholic solution of gum mastic (10 per cent) long enough to allow thorough infiltration (about 30 seconds).

7. Immerse for a moment in 95 per cent alcohol.

8. Wash in distilled water.

9. Silver nitrate (1 per cent aqueous solution) at 55° C. for from 1 to 6 hours without exposure to light. I find 4 hours best.

10. Wash for a moment in distilled water.

11. Develop for from 5 to 15 minutes in:

Hydroquinone	-----	1.5	Gm.
Sodium sulfite	-----	0.25	Gm.
Formaldehyde (neutral, Merck)	-----	10	c.c.
Acetone	-----	10	c.c.
Pyridine	-----	10	c.c.
Distilled water to make	-----	90	c.c.

Mix and dissolve these, and then add 10 c.c. of 10 per cent absolute alcoholic solution of gum mastic.

12. Wash for a moment in distilled water.

13. Dissolve the mastic and dehydrate in 95 per cent alcohol, then acetone. Clear in xylol and mount in gum dammar.

*Dieterle, R. R.: Arch. Neurol. & Psychiat. 18: 73, 1927.

To make mastic solution, dissolve 10 Gm. gum mastic in 100 c.c. absolute alcohol. It takes 3 days to dissolve.

Always handle the sections with a glass rod.

Do not allow too much mastic to remain in the sections. After the silvering, about 2 dips in successive waters are enough to prevent too dark a stain of the entire section.

Spirochetes appear jet black with yellow background.

This is an excellent method and can be highly recommended.

Krajian's Rapid Method for Staining Spirochaeta Pallida in Single Tissue Sections*

Improved Technic

Drop fresh blocks of tissue, about 5 mm. thick, in a 10 per cent solution of formaldehyde heated to 67° C. (152.6° F.) and fix in an oven at 67° C. for 10 minutes. (Tissues already fixed with formaldehyde do not require this treatment.)

1. Cut thin sections, from 5 to 10 microns, on a freezing microtome.

2. Wash sections in distilled water.

3. Place in the following (No. 1) solution for 15 minutes at 67° C.:

Uranium nitrate -----	1 Gm.
Formic acid, C.P., 85 per cent -----	3 c.c.
Glycerin, C.P. -----	5 c.c.
Acetone, C.P. -----	10 c.c.
Alcohol, 95 per cent -----	10 c.c.

4. Wash in 2 changes of distilled water.

5. Dip in a dilute gum mastic solution for 5 seconds (3 drops of saturated alcoholic solution of gum mastic in 5 c.c. of 95 per cent alcohol).

6. Rinse in distilled water.

7. Place in a 0.75 per cent aqueous solution of silver nitrate for 1 hour (previously warmed to 67° C.).

8. Without washing, place in about 3 to 5 c.c. of the developing solution, which prior to use is warmed to 60° C. While developing, work under a 60 watt electric lamp, 4 feet above the specimen.

Dip section in and out and expose to the electric light 6 to 8 times, or until completely brown.

*Krajian, A. A.: Am. J. Syph., Jan., 1933, and Arch. Dermat. & Syph., Nov., 1935.

The composition of developing solution :

Hydroquinone -----	0.31 Gm.
Sodium sulfite -----	0.06 Gm.
Acetone -----	2.5 c.c.
Formaldehyde, 40 per cent -----	2.5 c.c.
Pyridine -----	2.5 c.c.
Saturated solution of gum mastic in 95 per cent alcohol -----	2.5 c.c.
Distilled water -----	15 c.c.

9. Rinse in 95 per cent alcohol.

10. Rinse in distilled water.

11. Place in 0.75 per cent solution of silver nitrate for from 15 to 25 seconds.

12. Wash in distilled water.

13. Draw sections onto slides and blot with fine filter paper.

14. Dehydrate with absolute alcohol or anhydrous isopropanol for 1 minute, then blot and dip in thin celloidin.

15. Clear in pure xylol for 2 minutes.

16. Mount in gum dammar.

Comment.—The sections should be cut quite thin, preferably from 5 to 10 microns in thickness, as thicker sections make demonstration of spirochetes difficult owing to the fat content.

The purpose of using acetone and alcohol in No. 1 solution is to remove any fatty substance present in tissue, especially in sections of liver and brain.

The temperature of the oven is important; 67° C. has proved to be optimum. Lower temperatures require a longer time, while 70° C. or above has resulted in failure to demonstrate spirochetes in known control tissue.

Both the No. 1 solution and the solution of silver nitrate should be warmed to 67° C. before sections are placed in them. The No. 1 solution requires 15 minutes to be heated to 67° C., and the solution of silver nitrate about 30 minutes. In order to save time, therefore, it is a good practice to place the solution in the oven before one begins to cut sections.

Control tissues should always be prepared from material known to contain spirochetes.

The developing solution, when made up fresh, has a creamy color, but soon turns brown. The solution keeps well from 2 to 3 weeks in a light room. After that time it begins to deteriorate, the mastic

separating and settling to the bottom of the container. When this occurs, a fresh supply should be prepared.

The No. 1 solution is stable and keeps indefinitely.

Saturated gum mastic is prepared by dissolving 25 Gm. of resin in 30 c.c. of absolute, or 95 per cent ethyl, alcohol. Only clear portion of mastic should be used for developing solution.

Examination of Sections.—In primary lesions it is comparatively easy to find spirochetes in large numbers, and generally one section is sufficient for examination. With chronic lesions, half a dozen or more sections should be prepared and carefully searched.

A Twenty-Minute Staining Method for Spirochaeta Pallida in Tissue Sections*

The method:

1. Boil 10 per cent formaldehyde in Pyrex test tube, drop small biopsy material in it, and agitate for 1 minute. Regular formal-fixed tissues do not require this treatment.

2. Cut frozen sections 7 to 10 microns.

3. Place sections in No. 1 solution (previously heated to 60° C.) for 5 minutes and keep in paraffin oven or water bath registering 56° to 60° C.

Composition of the solution:

Uranium nitrate -----	1 Gm.
Formic acid, 85 per cent -----	3 c.c.
Glycerin (C.P.) -----	5 c.c.
Acetone -----	10 c.c.
Alcohol, 95 per cent -----	10 c.c.

4. Rinse in distilled water.

5. Treat for 5 seconds in a dilute gum mastic solution prepared as follows:

Mix well 3 drops of saturated alcoholic solution of gum mastic in 5 c.c. of 95 per cent alcohol.

6. Rinse and spread out section in distilled water.

7. Place in a wide mouth Pyrex beaker or other suitable Pyrex receptacle containing 30 c.c. of a 1 per cent silver nitrate solution.

8. Heat the silver solution with a Bunsen burner until bubbles form. Place thermometer in the receptacle and keep temperature at about 70° C., heating solution with burner every few seconds for 7 minutes.

*Krajian, A. A.: Am. J. Syph., Gonorr. & Ven. Dis. 23: 617-620, 1939.

9. Without washing, carry individual sections with a glass rod lifter to the warm developing solution, alternately dipping into the solution and then exposing all portions of the section on the lifter to a strong electric light, repeating this process 6 to 8 times or until section assumes brown color.

The developing solution:

Hydroquinone -----	0.31 Gm.
Sodium sulfite -----	0.1 Gm.
Formaldehyde, solution 40 per cent -----	2.5 c.c.
Acetone -----	2.5 c.c.
Pyridine -----	2.5 c.c.
Saturated alcoholic solution of gum mastic ----	2.5 c.c.
Distilled water -----	15 c.c.

10. Wash for a few seconds in 95 per cent alcohol.

11. Rinse in distilled water and spread out section by surface tension. If folded over, continue treatment with alcohol further until completely smooth.

12. Again place in silver solution for 10 to 20 seconds and rinse in distilled water.

13. Place in a large basin of distilled water and transfer to glass slide. (Section being of brown color should have a white background under basin.)

14. Dehydrate for 30 seconds with absolute alcohol or anhydrous isopropanol.

15. Blot twice with filter paper and dip in thin celloidin once. Wipe the back of slide with clean towel. Isopropanol for several seconds.

16. Place in neutral xylol for 1 minute. Blot twice and place in xylol for a few seconds.

17. Mount in gum dammar. Place identification number or name of the patient under cover slip for permanent record.

The No. 1 solution is stable and keeps indefinitely. A 10 per cent stock solution of silver nitrate is kept in a dark cool place from which 1 per cent solution is prepared. The stock solution keeps well.

Dilute gum mastic should be prepared fresh. Saturated gum mastic is prepared by dissolving 25 Gm. of resin in 35 c.c. of absolute alcohol. Shake the solution about 3 times a day for 3 to 5 days until clear. It is not necessary to filter; use only clear portion.

Developing solution keeps well about 2 weeks in a light room, after which it deteriorates, the gum mastic separating and settling to the bottom of the container. When this occurs, a fresh supply should be prepared.

The developing solution should be warmed to 60° C. before use.

The silvering and developing should be done under 60 watt electric light, about 4 feet above specimen.

This is the shortest method of staining *Spirochaeta pallida*.

Clinical Application of a Rapid Staining Method for the Identification of *Spirochaeta Pallida* in Paraffin Sections

Stokes* states that the universal adoption of dark-field method for the early diagnosis of syphilis is caused by the exceptional difficulty and delay in staining methods. However, it is admitted that there is an urgent need for a rapid staining technic to replace the dark-field procedure for the identification of *Spirochaeta pallida*, for upon it depends our best hope for the control and extinction of this disease as a public health problem.

Teaching dark-field technic to medical or laboratory students has convinced many that the use of dark field as an instrument for early diagnosis has theoretical rather than practical value. That even laboratories and individuals claiming to recognize the characteristic morphology of *S. pallida* require supervision, is evident from complaints with reference to the work of commercial laboratories, and experiences of syphilis clinics which receive diagnosis made in this way by outside sources.

The identification of *S. pallida* by dark-field technic is also a problem. Because of the common location of the primary syphilitic lesions, saprophytic spiral forms, which are normal inhabitants, cause great confusion, particularly of the material from mouth and anal lesions. Many students of spirochete have stated that they are unable to identify *S. pallida* definitely in the presence of other spiral form organisms, and therefore refuse to make diagnosis.

Since the saprophytic types are surface inhabitants, it seems obvious that the biopsy method completely avoids the confusion forms, because *S. pallida* are found deep in the tissue where they re-

*Stokes, J. H.: Modern Clinical Syphilology, Philadelphia, 1934, W. B. Saunders Co.

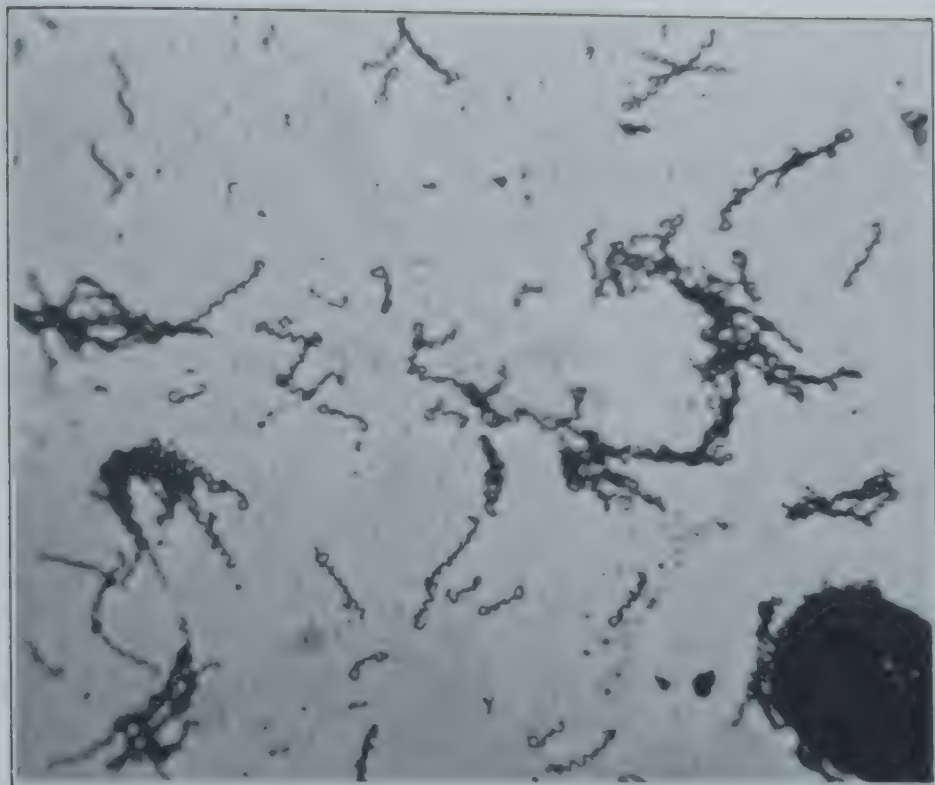


Fig. 106.—*Leptospira icterohaemorrhagiae*. Smear preparation from culture.
Stained by Krajian method.

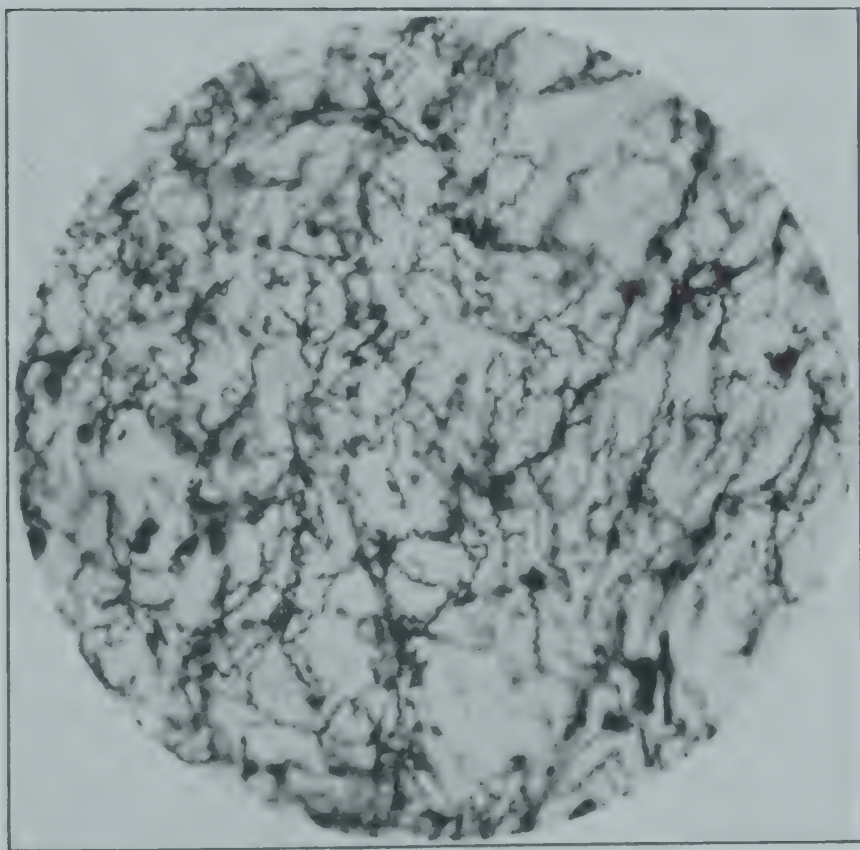


Fig. 107. *Spirachta pallida* in liver section from a case of congenital syphilis.
Stained by Krajian method.

tain their characteristic form. It is stated by Noguchi and Stitt, that *S. pallida* is the only spirochete that retains its characteristic form after death. Therefore finding of morphologically characteristic spiral form organisms deep in the tissue makes the diagnosis much simpler and reliable.

Syphilis is a public health problem, especially during the world wars, and it is our belief that the biopsy method could advantageously be employed in the general program against this disease by

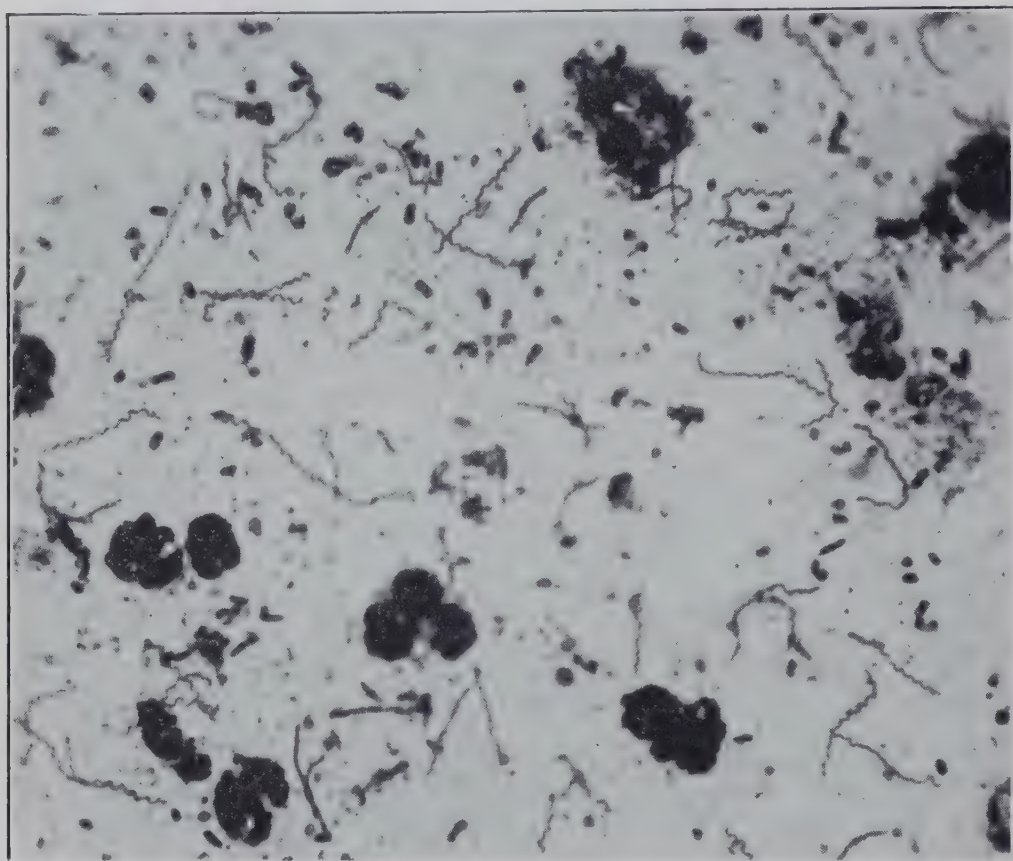


Fig. 108.—*Spirochaeta pallida* in a section from primary lesion. Stained by Krajan method.

the U. S. Public Health Department, by departments of health of states, counties, municipalities, and well-equipped hospital and commercial laboratories, where the technic is carried out by technicians and the microscopic preparations examined by the pathologist or bacteriologist.

The simplicity of the method is attested by the fact that student technicians have had no difficulty in producing well-stained preparations at first attempt. The reliability of the method has also

been adequately checked both clinically and serologically and controlled by using tissues known to contain *S. pallida*.

A rapid method for the demonstration of *S. pallida* in frozen sections was described previously.*

For the benefit of those who still prefer to employ the paraffin method for the preparation of microscopic sections, recently I have devised a rapid method of staining *S. pallida* in paraffin sections which requires 40 minutes to perform after sections are cut. The primary fixation of tissue and embedding process will require about 4 hours.



Fig. 109.—*Spirochaeta pallida* in skin lesion. Secondary syphilis, stained by Krajan method.

The method (primary fixation and embedding) :

1. Place thin blocks of biopsy or autopsy material in warmed (about 40° C.) 10 per cent formaldehyde and fix in incubator for 30 minutes.

2. Rinse in tap water and place in acetone-alcohol (1 part of acetone plus one part of 80 per cent alcohol) for 30 minutes.

*Krajan, A. A.: Am. J. Syph., Gonorr. & Ven. Dis. 23: 617-620, 1939.

3. Discard and place in pure acetone for 30 minutes.
4. Discard and place in chloroform for 30 minutes.
5. Discard and place in chloroform-paraffin mixture (equal parts of chloroform and melted paraffin) in paraffin oven at 56° C. for 30 minutes.
6. Place in pure paraffin in paraffin oven for 30 minutes.
7. Place in second paraffin bath for 30 minutes in paraffin oven.
8. Embed in fresh paraffin and place in ice water to harden.
9. Cut sections 5 to 7 microns and immediately drop in a small staining dish containing about 10 c.c. xylene.



Fig. 110.—*Spirochaeta pallida* in gumma of testicle. Tertiary lesion, stained by Krajan method.

Staining:

1. Pour about 20 c.c. of 95 per cent alcohol or absolute ethyl alcohol in xylene dish containing the sections, and within 30 seconds transfer to about 10 c.c. fresh 95 per cent alcohol.
2. Place sections in No. 1 solution (mordant) previously heated, and leave in paraffin oven at 56° C. for 10 minutes.

3. Rinse in distilled water and dip in and out 3 times in dilute gum mastic solution.

4. Rinse in distilled water and place in a 250 c.c. capacity beaker, containing 40 c.c. of 1 per cent silver nitrate solution.

5. Place beaker on a metal warming table, previously heated with a Bunsen burner, until bubbles form (about 70° C.) and keep at that temperature for 15 minutes. This silvering process should be done under electric light (60 watts) about 4 feet above the specimen.

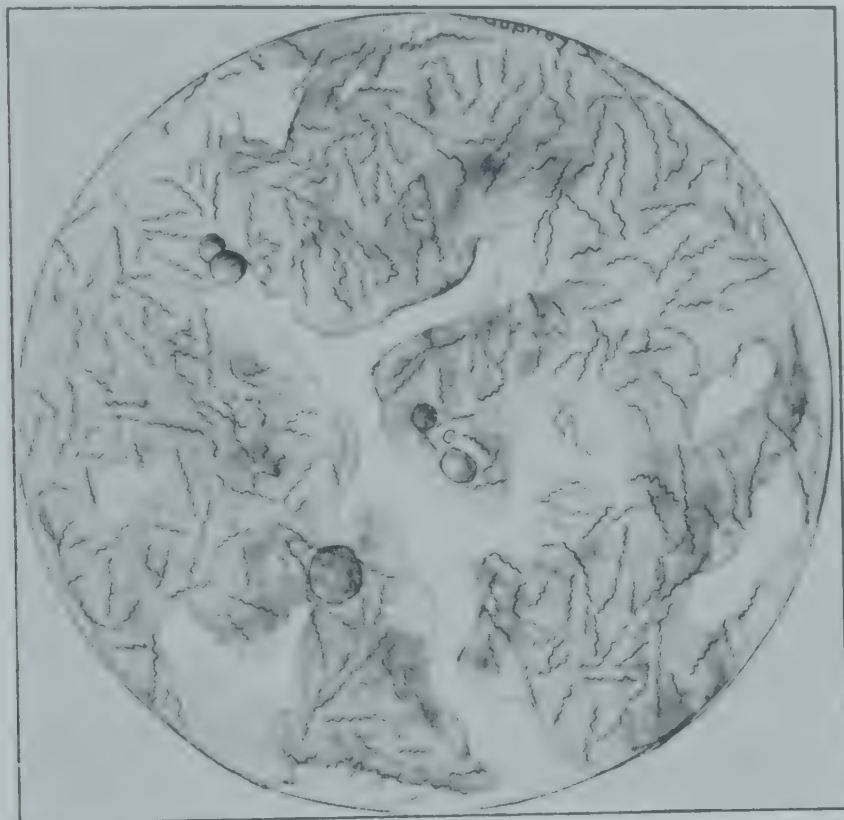


FIG. 111.—*Spirochaeta pallida*. Drawing from a case of congenital syphilis of liver. Section stained by Krajian method.

6. Develop in a hot developing solution, previously heated in paraffin oven, dipping section in and out several times and exposing to electric light until dark brown.

7. Place in about 10 c.c. of 95 per cent alcohol to remove the gum present in the developing solution.

8. Transfer once more to the beaker containing the used silver nitrate solution for 1 minute (this makes the spirochetes jet black).

9. Place in distilled water, and transfer sections on glass slide (float sections in a large basin containing tap water, placing white paper or towel under paper to aid in seeing the colored sections).

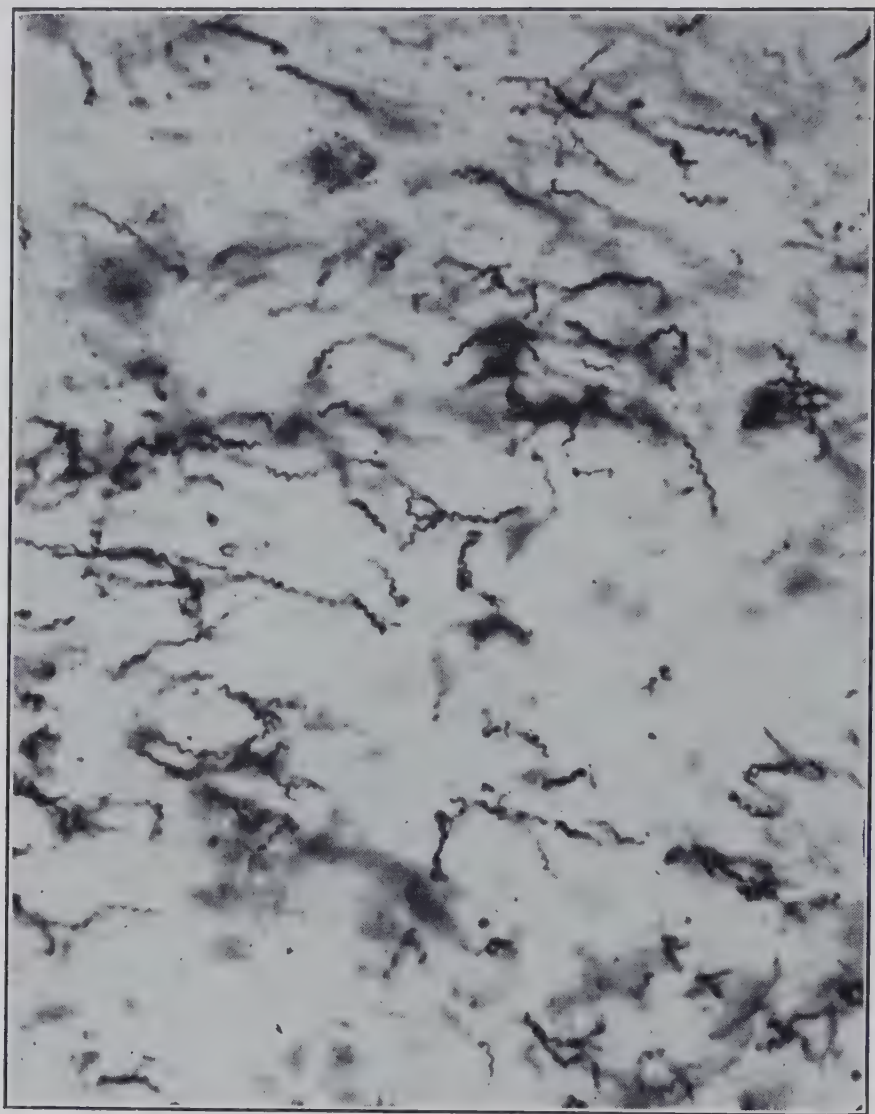


Fig. 112.—A case of syphilitic aortitis. Note various forms of *Spirochaeta pallida*, especially broken and granular types. Stained by Krajan method.

10. Blot immediately in fine filter paper and dehydrate, pouring several drops of anhydrous isopropanol or absolute ethyl alcohol from a drop bottle, pour off, blot and dip once in thin celloidin, blow breath over the section until dry, blot and rehydrate by pouring several drops of absolute alcohol or isopropanol.

11. Flood section with creosote-xylene (1 part beechwood creosote, 2 parts xylene) for 3 minutes.

12. Discard and flood with pure xylene for 3 minutes and mount in gum dammar.

Composition of the solutions:

No. 1 Solution (Mordant)

Uranium nitrate -----	1 Gm.
Formic acid (85 per cent) -----	3 c.c.
Chloral hydrate -----	5 Gm.
Glycerin, C.P. -----	5 c.c.
Acetone -----	10 c.c.
Alcohol, 95 per cent -----	10 c.c.

Dilute gum mastic:

Add several drops of saturated alcoholic solution of gum mastic to 10 c.c. of 95 per cent ethyl alcohol until the mixture is milky.

Saturated alcoholic solution of gum mastic is prepared by adding 30 c.c. of absolute ethyl alcohol to 35 Gm. of gum mastic (resin) in a 2 oz. capacity specimen bottle and shaking several times a day until clear.

Developing Solution

Hydroquinone -----	0.3 Gm.
Sodium sulfite -----	0.1 Gm.
Formaldehyde (40 per cent) -----	2.5 c.c.
Acetone, C.P. -----	2.5 c.c.
Pyridine -----	2.5 c.c.
Gum mastic (saturated alcoholic solution) ----	2.5 c.c.
Distilled water -----	15 c.c.

Completely dissolve hydroquinone and sodium sulfite in formaldehyde and acetone; then add pyridine and gum mastic; mix well; finally add water and invert for thorough mixing.

Place the No. 1 solution (about 10 c.c.) and developing solution (about 5 c.c.) in small staining dishes with covers on, in paraffin oven at 56° C. Then heat the metal warming table with a lighted Bunsen burner under a 60 watt electric light. Next transfer sections from xylene to alcohol and from alcohol to warmed No. 1 solu-

tion which again is placed in paraffin oven. Next prepare the silver solution, taking 3 c.c. of a 10 per cent stock silver nitrate and mixing with 27 c.c. distilled water in a clean 250 c.c. capacity Pyrex beaker. Next prepare the dilute gum mastic, adding several drops of saturated alcoholic solution of gum mastic to about 10 c.c. of 95 per cent alcohol until milky.

No. 1 solution is stable and keeps indefinitely.

A 10 per cent stock solution of silver nitrate is kept at room temperature exposed to light, from which 1 per cent solution is prepared. The stock solution keeps well.

Dilute gum mastic is efficient as long as the gum is in suspension but when gum separates and settles to the bottom, a fresh dilution should be prepared.

Developing solution keeps well about 2 weeks in a light room, after which it deteriorates, the gum mastic separating and settling to the bottom of the container. When this occurs, a fresh supply should be prepared.

Morphological Variations of *Spirochaeta Pallida*

The morphological variations of *Spirochaeta pallida* are best observed in tissue sections and smears.

The typical form is one with regular, delicately curved, corkscrewlike, coil spring type and knotty type, and atypical forms are irregular in size and shape.

In tissue they are longer and thinner, especially when in large numbers (in very early lesions), when they are forced to struggle for existence. They get thicker and shorter when certain obstacles arise such as slight administration of oxygen, or being thrown in pus or exudate.

Schaudinn and others have advanced the idea that spirochetes have a quiescent stage, during which they change their morphology. Recently numerous other investigators have tried to confirm this hypothesis by observing knots, buds, wirelike, ringlike, granular, fragmented, sawlike, stretched, and other unusual forms.

Most investigators believe these atypical forms are part of the evolutionary cycle of the organism; others believe them to be the products of degeneration. These degenerative and atypical forms are generally seen in gummas where one sees extensive destruction of the tissue, where one will observe many peculiar forms, small particles, or remnants of the destroyed organisms. These peculiar

forms are generally observed in stained smears or tissue sections, where there has been a delay in fixation for several days, in the experimental animal; also they are seen in unfixed syphilitic human organs. The above findings suggest that the atypical forms are the product of degeneration.

1. Tightly woven Spirochetes with tapering ends, as seen in congenital syphilis.



2. Long and delicate forms, as seen in primary lesions.



3. Coil spring type, as seen in condylomas.



4. Knotty type, as seen in umbilical cords.



5. Atypical forms, broken and granular, as seen in tertiary lesions (gummas).



FUNGUS STAINS

Actinomycosis Group

It is generally believed that the filaments of the actinomycosis group, when stained by Gram-Weigert method, retain the gentian violet color. While we were examining biopsies from various skin lesions to demonstrate *Spirochaeta pallida* by the Krajan method, we encountered several cases which showed filaments of streptothrix. The same sections when stained by Gram-Weigert method gave negative results. Thus we concluded that some of these filaments belong to the gram-negative group, and found it advantageous to use both methods in suspected cases.

Coccidioides and Blastomyces

These organisms are easily stained and demonstrated by the regular hematoxylin-eosin method. (See Figs. 113 and 114.)

PIGMENT STAINS

The various pigments found in the human body under normal and abnormal conditions may be roughly divided into three groups, according to their origin:

- I. Hematogenous pigments.
- II. Autogenous pigments.
- III. Exogenous pigments.

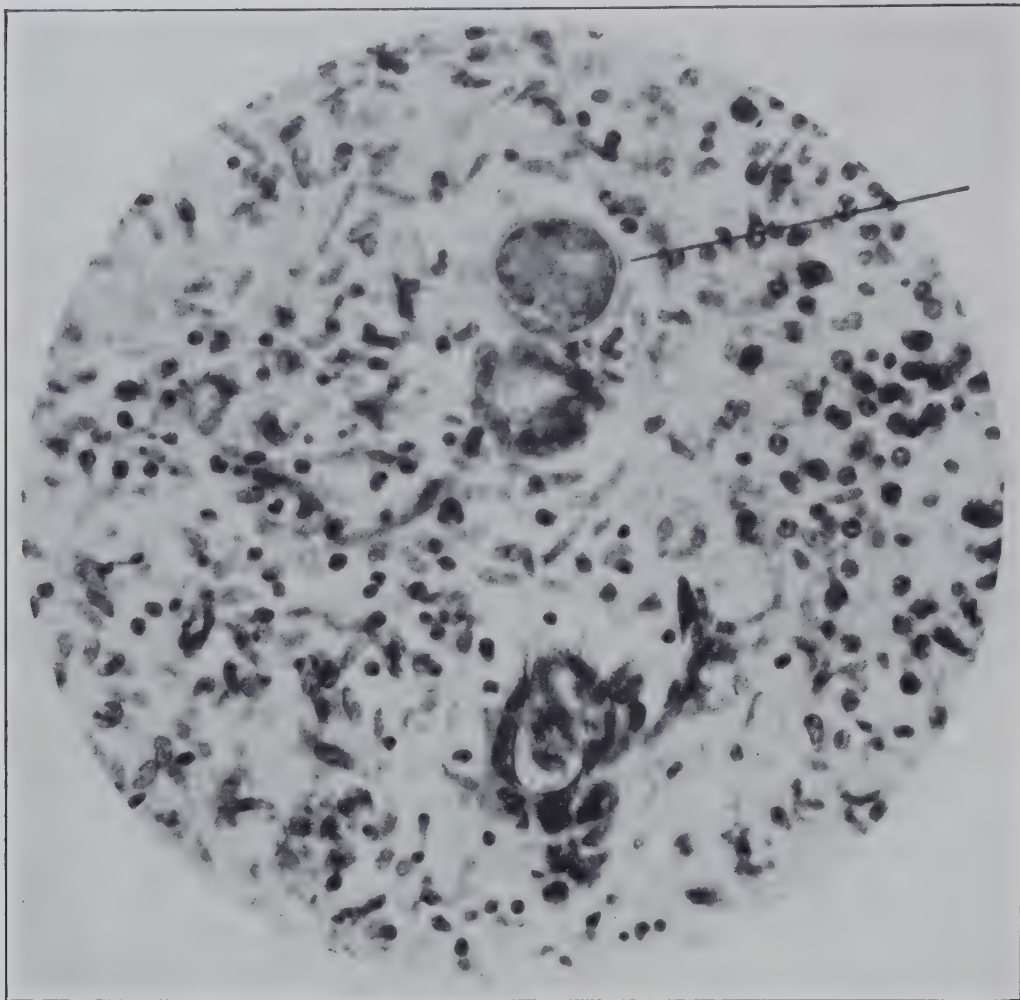


Fig. 113.—Coccidioides. Hematoxylin-eosinol stain. Frozen section. Line leads to coccidioides.

I. Hematogenous Pigments

Hematogenous pigments occur in considerable variety, but from the pathological standpoint, hemosiderin is the most important.

Hemoglobin occurs as yellowish-brown granules and droplets. It is soluble in water and dilute alcohol, but not in absolute alcohol.

Hematin occurs as a dark brown or bluish-black pigment. It has been stated by Brown that the pigment produced by malarial parasites is hematin.

Hemofuscin occurs as light yellow granules. It does not give the iron reaction with potassium ferrocyanide, is not soluble in hydrogen peroxide, and is found in liver and other organs in hemochromatosis.

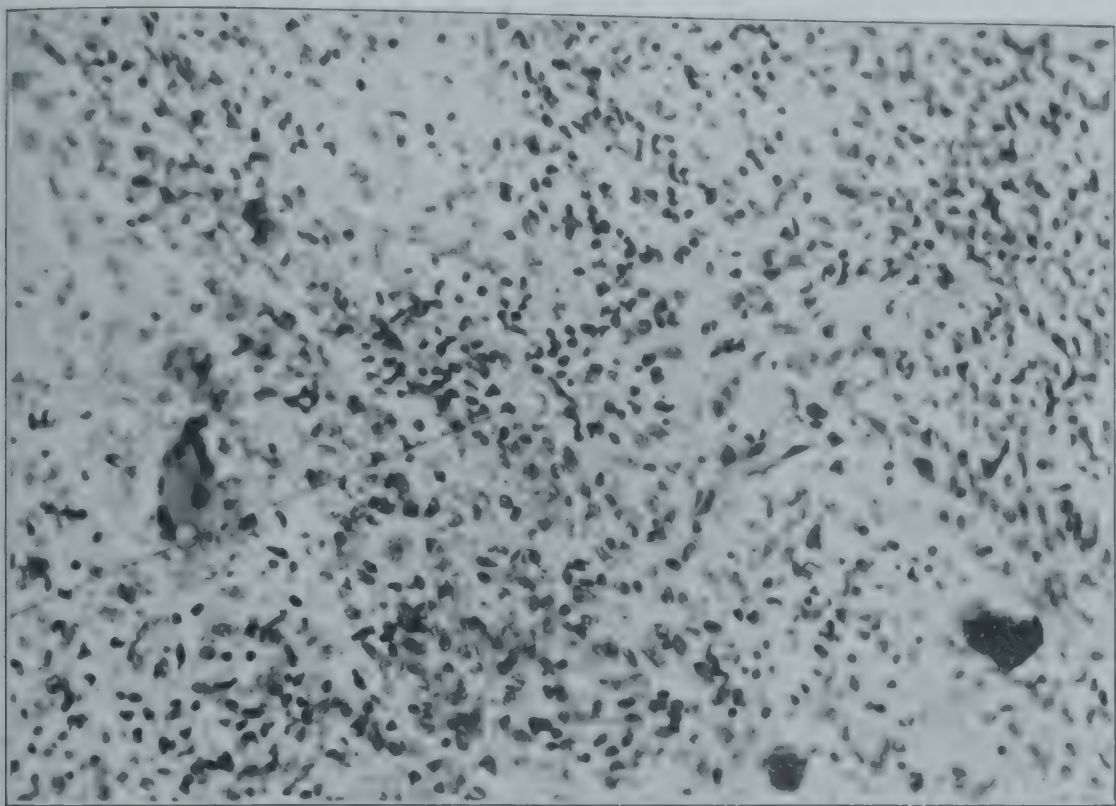


Fig. 114. Blastomyces in biopsy section. Hematoxylin-eosin stain. Frozen section. Line leads to Blastomyces.

Mallory's Fuchsin Stain for Hemofuscin

Fix tissues in Zenker's fluid, alcohol, or formaldehyde, and make paraffin, celloidin, or frozen sections.

1. Stain sections in Harris' hematoxylin until the nuclei stand out sharply.
2. Wash in tap water.
3. Stain 10 to 20 minutes in the following solution:

Fuchsin, basic	-----	0.3 Gm.
Alcohol, 95 per cent	-----	50 c.c.
Water	-----	50 c.c.

4. Wash in water.
5. Differentiate and dehydrate in 95 per cent alcohol, followed by absolute alcohol.
6. Clear in xylol and mount in colophonium. Nuclei stain blue; hemofuscin granules, bright red; hemosiderin remains unstained.

This is a very useful hemofuscin stain.

Hemozoin occurs as black granules formed by malarial parasites living in red blood corpuscles; it can be distinguished from carbon

by its solubility in concentrated sulfuric acid. Hemosiderin pigments do not give iron reaction.

Hemosiderin occurs as bright yellowish-brown granules and masses. It is not soluble in water, alcohol, ether, or alkalies, but is soluble in acids.

It does not stain with fat stains.

Iron Reaction With Potassium Ferrocyanide

1. Make frozen, paraffin, or celloidin sections in usual manner, and stain for 10 to 20 minutes in the following mixture:

Potassium ferrocyanide, 2 per cent aqueous solution, freshly prepared	1 part
Hydrochloric acid, 1 per cent	3 parts

Much better results will be obtained if the reaction is hastened by warming the fluid. Heat the mixture in a clean Pyrex test tube until beads of vapor form on the inner surface of the glass (about 70° C.). Then place the sections in it, or pour the mixture over them in a Stender dish. The reaction takes place within 1/2 to 2 minutes.

2. Wash thoroughly in several changes of distilled water. Fix the sections to the glass slide with thin celloidin.

3. Counterstain in eosin. Rinse in water.

4. Dehydrate completely with 2 changes of absolute alcohol.

5. Clear in pure xylol.

6. Mount in gum dammar.

Clear celloidin sections in oil origanum.

Use distilled water for solutions and washings.

Nuclei and hemofuscin stain bright red; hemosiderin, blue.

Stain for Gross Specimens Containing Iron

The potassium ferrocyanide and hydrochloric acid method may easily be applied to slices of fresh tissue for diagnostic purposes or after fixation. Attractive museum preparations may be made and preserved.

For Fresh Tissues

Thin slices of tissue are cut from suspected iron-containing organs and placed in the potassium ferrocyanide and hydrochloric acid mixture. If positive, the blue reaction takes place within 2 to 10 minutes.

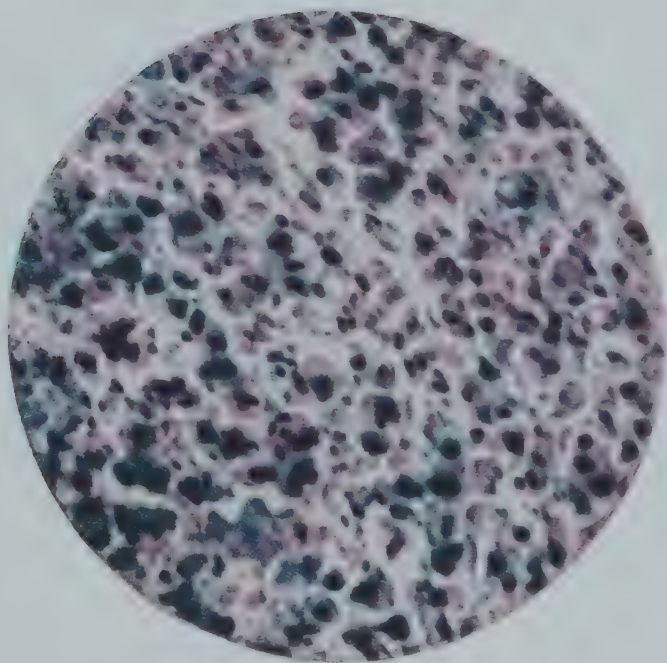


PLATE VII

Liver section from a case of pernicious anemia, stained by potassium ferrocyanide method. Iron pigments, dark blue; background, pink. Frozen section.

Magnification 250 diameters. Original on Kodachrome. Made with Bausch & Lomb 8.3 MM apochromatic objective of 0.65 N.A. and 7.5X compensating eyepiece.

For Museum Specimens

1. Fix large slices of liver or other organs containing iron in Kaiserling solution No. 1 for several days.
2. Wash thoroughly under running water overnight.
3. Preserve in 80 per cent alcohol.
4. Cut into thin slices in order to obtain smooth surfaces.
5. Place the slices into freshly prepared potassium ferrocyanide solution warmed to 70° C. The reaction takes place in 2 to 5 minutes.
6. Wash under running water for 24 hours.
7. Mount in 80 per cent alcohol along with unstained slice for contrast. (Do not use Kaiserling solution No. 3 as it causes the blue color to diffuse.)

This is a very useful method for museum specimens.

Hematoidin occurs as orange colored or red rhombic plates, or as radiating bunches of yellow needles. It contains no iron, it is not soluble in water, alcohol, or ether, but dissolves in chloroform. It is found in old hemorrhages, especially in infarcts of the spleen and brain.

Bile pigments occur as yellowish granules and masses. They are not soluble in water, alcohol, or ether.

II. Autogenous Pigments

Autogenous pigments occur normally in hairs and in the deeper layers of the epidermis, most abundantly in people of dark complexion. These pigments produced in the body are termed *melanin*.

Melanin is a dark brown substance of great coloring power, which occurs in cells as brown or black granules. It contains no iron or fat and does not react with iron or fat stains. It is not soluble in ordinary reagents except in alkalies. It is stained black by silver nitrate method.

Melanin Stain

(Masson's Method)

Make frozen, paraffin, or celloidin sections in the usual manner.

1. Wash sections in distilled water that is slightly ammoniacal (1 drop ammonium hydrate to 100 c.c. distilled water) for 2 to 3 hours.

2. Transfer sections into liquid of Fontana for 2 to 3 hours. The solution is made up as follows:

Take 5 per cent aqueous solution of silver nitrate and add ammonium hydrate until the precipitate which is formed is just dissolved. Then add 5 per cent silver nitrate drop by drop until the liquid is just opalescent. Let the fluid clear by standing.

3. Wash in distilled water.

4. Place sections in toning solution for 10 minutes.

A.

Ammonium sulfocyanate, 2 per cent in water	----	3 c.c.
Sodium hyposulfite	-----	3 Gm.
Distilled water	-----	100 c.c.

B.

Gold chloride, 1 per cent aqueous solution	-----	100 c.c.
--	-------	----------

Mix equal parts of A and B just before using.

5. Wash in distilled water.

6. Wash for 1 minute in 5 per cent sodium hyposulfite solution.

7. Wash in several changes of distilled water.

8. Dehydrate in 95 per cent, then in absolute alcohol.

9. Clear in xylol and mount in gum dammar.

Melanin pigments assume black color.

Excellent stain for melanin.

III. Exogenous Pigments

Various substances enter into the human body and are deposited there as pigments. Among these the most important are carotin, carbon, iron, and silver.

Carotin is an orange yellow pigment derived from carrot, squash, and certain other vegetables. It is easily soluble in fats and fat solvents. It is not stained with fat stains.

Carbon occurs commonly and abundantly in the lungs and bronchial lymph nodes. Sometimes it is carried to the spleen and liver where its recognition is important. It must be distinguished from malarial pigment. Its distinguishing characteristics are its extreme black color and its insolubility in concentrated sulfuric acid in which all other pigments dissolve.

Silver appears brown or black; it is turned black by ammonium sulfide and is removed by a mixture of potassium ferrocyanide and sodium hyposulfite.

Arsenic Pigments*

Microchemical Detection of Arsenic in Tissue

1. The tissue is fixed in 10 per cent formaldehyde for from 24 to 48 hours.
2. It is then washed in running water for at least 6 hours.
3. The tissue is cut in small pieces, not over 2 mm. thick.
4. Pieces of the tissue are placed in a 1 ounce ground glass-stoppered bottle, and the bottle nearly filled with fresh neutral hydrogen sulfide solution. A small amount of petroleum is placed on the stopper which is inserted tightly and then bound down with a tape or cord. The bottle and contents are incubated between 70° and 80° C. for 4 days. Staining qualities may be better preserved if the tissue is incubated at a lower temperature for a longer time (56° C. for 6 days). The neutral hydrogen sulfide is changed daily.
5. Wash tissue under running water for from 6 to 12 hours.
6. Dehydrate by successive passages through 50, 70, 80, and 95 per cent alcohol, and absolute alcohol, each containing about 10 per cent ether.
7. Embed in celloidin or paraffin, and cut sections about 5 microns. Remove the embedding medium from the section in usual manner.
8. Place sections for 20 minutes in 10 per cent hydrochloric acid made up in 95 per cent alcohol. This dissolves out all sulfides except the arsenic trisulfide. However, it also seriously impairs the staining qualities of the tissue.
9. Wash sections in 70 per cent alcohol for 20 minutes and stain with hematoxylin and eosin.
10. Differentiate with 95 per cent alcohol.
11. Clear sections in oil of cloves. Remove the excess with xylol.
12. Mount in gum dammar.

Characteristics of Arsenic Trisulfide

On microscopic examination with high magnification, the individual crystals are nearly round or oval, but somewhat irregular. Their color is yellow with a faint greenish tinge. When exactly in focus, the crystals appear solid with a bright halo. If they are too near the objective, they have a heavy rim, a clear center, and a slight halo. If they are beyond the exact focus, they have a clear center, a rather faint rim, and little or no halo.

*Osborne, Earl D.: Arch. Dermat. & Syph., Dec., 1925.

A point worthy of mention is that the arsenic pigments do not clump. Only occasionally will two crystals be seen side by side. Some are intracellular.

This is a very useful method to demonstrate arsenic pigments in tissue sections.

Simple Technic for Dopa Reaction (Laidlow)*

For Recognition of Melanoblasts

Bloch's dopa reaction is a specific stain for melanoblasts and for myelogenous leucocytes. These cells are believed to contain an organized ferment which oxidizes dioxyphenylalanine (dopa) to melanin. The dopa-melanin colors the reacting cell black. This blackening of the cell is the dopa reaction.

Melanoblasts.—For a dopa reaction of melanoblasts, the tissue must be fresh. After death or excision of the tissue from the living body, the intracellular ferment soon diffuses into the surrounding tissue and is quickly destroyed by most fixatives and preservatives. The ideal material is a frozen section of fresh tissue made immediately after excision from the living body. However, it is difficult to cut fresh tissue neatly. In practice Bloch's present custom of hardening thin slices of freshly removed tissue in 5 per cent formalin for 2 to 3 hours is followed. After this short fixation the tissue cuts better, and the reaction is in no way impaired by the short stay in 5 per cent formalin. Neither the gross specimen nor the sections should be permitted to lie in water for more than a few seconds. Water and dilute alcohol extract the ferment rapidly.

The reagents required are a stock solution of dopa and the Sörensen buffers.

Stock Dopa Solution.—This is a 1:1,000 solution of 3,4-dioxyphenylalanine (abbreviated to dopa) in distilled water. Dopa† is a phenol extracted from *Vicia faba*, a common vetch or soybean. The levorotatory preparation should be used, since Bloch, and Schaaf and Peck and co-workers, have shown that melanoblasts have little or no oxidizing power over the dextrorotatory form. In powder, as purchased, dopa keeps indefinitely at room temperature.

Dissolve 0.3 Gm. dopa powder in 300 c.c. cold distilled water. Keep well corked in the refrigerator, where it will remain stable

*Am. J. Path. 8: 491-498, 1932.

†When ordering, specify "for Bloch's dopa reaction." Supplied by the American branch of Hoffmann-La Roche, Nutley, N. J.

for many weeks. The solution is usable as long as it is colorless or only slightly tinged with red. Darker red solutions should be rejected; they oxidize too quickly and overstain the sections.

The Buffers.—Dissolve 11 Gm. of disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) in 1,000 c.c. of distilled water.

Dissolve 9 Gm. potassium dihydrogen phosphate (KH_2PO_4) in 1,000 c.c. distilled water. Both of these buffers are kept in the refrigerator.

Just before cutting the sections, buffer to 7.4 by adding 2 c.c. potassium phosphate and 6 c.c. sodium phosphate buffer to 25 c.c. of the stock dopa solution. For a small batch of a dozen sections use 15 c.c. of the buffered solution, but prepare double the quantity immediately required in order to have enough to renew the solution in half an hour. Return the stock dopa solution and the surplus of the buffered solution to the refrigerator immediately; at room temperature the stock solution soon oxidizes and turns red; the buffered solution tends to turn brown.

At a given temperature the speed of the reaction is determined by the pH. At 7.4 the reaction will be finished in 4 or 5 hours at 37°C . When in a hurry, hasten reaction by using only 1 c.c. of the potassium buffer, giving a pH of 7.7, or omit the potassium buffer, obtaining a pH of 8.2. Such solutions react quickly, in about 60 minutes at 37°C . They should be inspected every 20 minutes to forestall overstaining. These hurried reactions are likely to be overstained; the slow reactions give much more delicate pictures.

A trace of acid inhibits the reaction; a trace of alkali hastens it. All glassware, therefore, should be scrupulously and chemically clean.

Cutting the Sections.—Frozen sections are obligatory, since the chemicals of celloidin and paraffin embedding will destroy the ferment. It is important to remember that water extracts the ferment quickly. Neither the block nor the sections should lie in water longer than the few seconds of a quick rinse. Before cutting the sections, the dopa solution should be buffered and poured into dishes ready to receive the sections without delay.

In order to exhibit the long dendrites of melanoblasts, some of the sections should be very thick, 75 to 100 microns; others may be between 20 and 30 microns for better detail. Sections of fresh tissue are dropped from the knife directly into dopa. If the tissue has been in formalin, the sections are rinsed for a few seconds in dis-

tilled water and placed promptly in the dopa solution. Since the reaction is an oxidation, the dish is left uncovered for free access of air.

Staining.—The dish of dopa containing the sections is put in the incubator at 37° C. for about half an hour. Then the fluid is replaced by fresh solution, which in the meantime has been kept cold in the refrigerator.

After the first dopa is replaced with fresh solution, the reaction is inspected every half hour. In 2 or 3 hours the fluid turns reddish, then sepia brown. The appearance of the sepia tint signals the end of the reaction. At this point a section is rinsed and examined under the microscope.

In the perfect reaction the bodies of the dopa-positive cells (melanoblasts and leucocytes) are gray or black, melanin retains its natural yellowish-brown color, and collagen is colorless or the palest shade of gray. If a darker stain of melanoblasts is desired, the section is returned to the dopa solution for another half hour or so. The reaction finished, wash the sections in water, dehydrate, clear, and mount in balsam or gum dammar.

As a counterstain Bloch and dermatologists generally use methyl green pyronine. Laidlow prefers cresyl violet well differentiated with alcohol, as giving a paler ground. All counterstains take better if the dopa sections are first dehydrated, cleared, and brought back through alcohol to water.

Carcinoids (Argentaffin Cell Stain) (Masson)*

Technic

Fixation in either of the following:

Bouin's Fluid

Formaldehyde, 40 per cent	10 c.c.
Water	30 c.c.
Glacial acetic acid or 10 per cent trichloracetic acid	2 c.c.
Picric acid	to saturation

Optimum fixation time: 3 days.

Do not wash in water. Dehydrate immediately in alcohol and embed in paraffin.

*Am. J. Path. 4: 181, 1928.

Regaud's Fluid

Formaldehyde, 40 per cent	20 c.c.
Potassium bichromate, 3 per cent aqueous solution ..	80 c.c.

Optimum fixation time: 24 hours.

Wash in running water. Embed in paraffin.

Sections: Sections of 5 microns should be numerous and in series. The lesions to be studied are always localized; we must multiply the chances of finding them and be able to study them in three dimensions. The buds and the neuromas cannot be understood without many serial sections.

Affixing the Sections.—Dissolve 0.5 Gm. gelatin (in practice, a bit of ordinary sheet gelatin $\frac{1}{4}$ inch square) in 20 c.c. distilled water, warming the water over the flame. Filter a few drops of this solution on the slide and float the section on it. Place the preparation on the warm plate at 40° C. As soon as the section spreads, remove the slide, hold the section in place with a brush or needle, and stand the slide upright to drain. Blot with absorbent paper and dry in the oven at 40° C. in formalin vapor secured by leaving in the oven an open bottle of formalin.

This is the only method which gives perfect adhesion of the sections no matter what the fixative, the duration of the stain, or the temperature employed.

Trichrome Stain

First Stage.—Staining the nuclei with iron hematoxylin.

The sections, freed from paraffin by toluol, alcohol, and water, are immersed in 5 per cent iron alum previously heated to 45° or 50° C. for 5 minutes. Wash in water.

Stain for 5 minutes at 45° to 50° C. in Regaud's hematoxylin.

Hematoxylin	1 Gm.
Alcohol, 95 per cent	10 c.c.
Glycerin	10 c.c.
Distilled water	80 c.c.

Rinse with 95 per cent alcohol.

Differentiate in picric alcohol, which is more selective than iron alum.

Alcohol, 95 per cent saturated with picric acid	2 parts
Alcohol, 95 per cent	1 part

Wash in running water.

Second Stage.—Staining the cytoplasm and the collagen.
Prepare the following solutions:

(A) Acid fuchsin -----	0.3 Gm.
Ponceau de xyloidine* -----	0.7 Gm.
Distilled water -----	100 c.c.
Glacial acetic acid -----	1 c.c.
(B) Phosphomolybdic acid -----	1 Gm.
Distilled water -----	100 c.c.
(C) Glacial acetic acid -----	2 c.c.
Distilled water -----	100 c.c.
Aniline blue -----	to saturation

Stain in A for 5 minutes.

Rinse with distilled water.

Differentiate in B for 5 minutes.

Without rinsing, pour 10 drops of C on the section, tilt the slide a few times for thorough mixing, and let stand for 5 minutes.

Rinse in distilled water.

Back to B for 5 minutes.

In 1 per cent acetic acid water for 5 minutes.

Dehydrate, clear, and mount in salicylic balsam.

Results: Nuclei stain black; argentaffin granules, black or red; cytoplasm and neuroglia, vermilion red; collagen, intense blue.

Silver Method

(A) Silvering the sections:

Fixation.—Contrary to the opinion of certain authors, we believe tissue that is to be treated with silver should not be fixed in potassium bichromate, which gives poor results. Formol preserves granules very well but delicate cytoplasmic structures very poorly. The picric-acetic formol of Bouin is the fixative of choice.

Prepare the ammoniacal silver nitrate solution as follows:

To 100 c.c. of a 20 per cent aqueous solution of silver nitrate, add ammonium hydroxide drop by drop, shaking well, until the precipitate of silver oxide is just dissolved; then add a few drops of the 20 per cent silver nitrate until there is a persistent opalescence. The fluid should have no odor of ammonia. Add distilled water to 200 c.c., making the resulting solution 10 per cent silver nitrate. Keep in a strictly clean glass bottle and filter just before use.

*Of the brand Microcolor, 35 rue Escudier, Bologne-sur-Seine (Seine), France. Other brands of ponceau are very inferior for this purpose.

The sections, affixed to slides with gelatin, are immersed in alcohol, alcohol, and distilled water; then in the ammoniacal silver nitrate at room temperature in the dark.

In 4 or 5 hours, the argentaffin granules turn yellow, then brown. After 24 hours, or in 36 hours at the longest, the silver is completely reduced. Within this time limit, the silver is reduced also on certain coarse granules sometimes contained in the macrophages of the reticulum, granules of lipofuscin or of purine products, all very different from the fine granules of the argentaffin cells. Too long immersion colors the nuclei and the connective tissue.

After immersion in the ammoniacal silver for 24 hours, or 36 hours at the longest, the sections are washed in water and toned in the gold bath as follows:

(A)	Ammonium sulfoeyanide -----	6 Gm.
	Distilled water -----	100 c.c.
(B)	Sodium hyposulfite -----	6 Gm.
	Distilled water -----	100 c.c.
(C)	Gold chloride -----	2 Gm.
	Distilled water -----	100 c.c.

Mix 1 c.c. A with 1 c.c. B; add C until there is coarse precipitation, and pour over the sections. Toning is instantaneous.

Rinse with 6 per cent hypo. Wash in running water.

Result: The argentaffin granules are opaque black.

It is well to stain the background with either Cajal's picroindigocarmin, or, better, with ponceau-acid fuchsin-phosphomolybdic acid-aniline blue, the second stage of trichrome staining. We recommend the latter especially, for it brings out all the tissue elements perfectly.

(B) Silvering in the block:

Fix in Bouin for 3 days.

Cut slices 2 to 3 mm. thick. Wash them in running water for 24 hours.

Immerse for 24 hours in a solution of 2 drops ammonium hydroxide in 100 c.c. distilled water.

Immerse for 24 hours in the ammoniacal silver nitrate diluted with equal volumes of distilled water.

Rinse in distilled water.

Tone in Cajal's mixture for 24 hours:

Ammonium sulfocyanide -----	3 Gm.
Sodium hyposulfite -----	3 Gm.
Distilled water -----	100 c.c.
Gold chloride, 1 per cent solution -----	1 c.c.

Wash in water for several hours. Embed in paraffin or celloidin.

All the argentaffin granules are opaque black; the nuclei are brownish. Counterstaining is unnecessary. The nerves are never impregnated by this method.

Argentaffin Cell Stain

(Krajian)

A Rapid Method for Frozen Sections

1. Cut thin frozen sections of formaldehyde-fixed tissue.
2. Place for 30 minutes in a 10 per cent solution of ammonium hydroxide at 56° C.

3. Impregnate for 2 hours at 55° C. in ammoniacal silver solution prepared as follows:

To 15 c.c. of 10 per cent aqueous solution of silver nitrate, add strong ammonium hydroxide (28 per cent) drop by drop, shaking well, until the precipitate of silver oxide is just dissolved; then add a few drops of 10 per cent silver nitrate until there is a persistent opalescence.

4. Rinse in distilled water.
5. Tone in 1:300 solution of gold chloride for 5 minutes.
6. Treat for 5 minutes in 5 per cent sodium hyposulfite solution.
7. Wash in distilled water.
8. Transfer sections to glass slides in usual manner.
9. Dehydrate with isopropanol or absolute alcohol.
10. Clear in xylol.
11. Mount in gum dammar.

Argentaffin granules stain opaque black; nuclei, light brown.

Calcium Salts

Calcification is the term applied to the infiltration of tissue with calcium phosphate and calcium carbonate. The salts appear under the microscope as small refractive granules which may be mistaken for fat. They are dissolved in 5 per cent nitric or hydrochloric acid.

Von Kossa's Silver Method

Fix tissues in neutral formaldehyde or alcohol, and make frozen, paraffin, or celloidin sections.

1. Place sections in a 1.5 per cent aqueous silver nitrate for from 10 to 60 minutes.
2. Wash in distilled water.
3. Counterstain nuclei in safranine.
4. Dehydrate in absolute alcohol.
5. Clear in xylol and mount in gum dammar.

Calcium salts appear as opaque black areas in section.

This is a very useful stain to demonstrate calcium in tissue.

Purpurin Method

Fix tissues in neutral formaldehyde or alcohol, and make paraffin, celloidin, or frozen sections.

1. Stain in a saturated solution of purpurin in 95 per cent alcohol for from 5 to 10 minutes.
2. Rinse in a 0.75 per cent solution of sodium chloride for from 5 to 10 minutes.
3. Differentiate in 70 per cent alcohol until the stain ceases to wash out of the section.
4. Dehydrate rapidly in absolute alcohol.
5. Clear in oil origanum.
6. Mount in gum dammar.

Calcium salts stain red.

This is a very useful method.

CHROMATIN STAIN

Fix tissues preferably in mercuric chloride-acetic acid. Embed in paraffin and cut sections in the usual manner.

1. Stain sections in Unna's pyronine methyl green stain for from 10 to 30 minutes:

Methyl green (pure) -----	0.15 Gm.
Pyronine -----	0.25 Gm.
Alcohol, 95 per cent -----	2.5 c.c.
Glycerin -----	20 c.c.
Phenol, 0.5 per cent aqueous solution -----	100 c.c.

The solution keeps indefinitely.

2. Wash in distilled water.

3. Blot in filter paper.
4. Dehydrate rapidly in absolute alcohol.
5. Clear in xylol.
6. Mount in gum dammar.

Chromatin and nucleoli are fairly selectively stained green. Cytoplasm and bacteria stain red.

Stain is not permanent.

CHROMAFFIN TISSUE STAINS

Wiesel Method

1. Fix tissues for from 1 to 4 days in the following fluid:

Potassium bichromate, 5 per cent aqueous solution	10 c.c.
Formaldehyde, 10 per cent solution	20 c.c.
Distilled water	20 c.c.

2. Treat with 5 per cent potassium bichromate for 1 to 2 days.
 3. Wash overnight under running water.
 4. Dehydrate, clear, and embed in paraffin.
 5. Stain sections for 20 minutes in a 1 per cent aqueous solution of toluidine blue or wasserblau.
 6. Wash in water for 5 minutes.
 7. Stain for 20 minutes in a 1 per cent aqueous solution of safranine.
 8. Treat with 95 per cent alcohol until the sections turn blue.
 9. Dehydrate in absolute alcohol.
 10. Clear in xylol. Mount in gum dammar.
- Chromaffin cells stain green; nuclei, red; cytoplasm of adrenal cortical cells, light blue.

Better results are obtained by omitting the use of safranin in Step 7 (Krajian).

Result: Chromaffin cells stain green; cytoplasm of adrenal cortical cells, light blue.

This is a dependable chromaffin stain.

Giemsa's Method

1. Fix tissues in formol-bichromate mixture as used in the Wiesel method.
2. Wash in running water.
3. Make frozen sections (said to be preferable) or embed in paraffin and make sections.

4. Stain sections for from several to 24 hours in Giemsa's stain (drop of Giemsa's for every cubic centimeter of neutral distilled water).

5. Wash in water.

6. Blot in fine filter paper.

7. Dehydrate with acid-free acetone and control differentiation under the microscope.

8. Clear in xylol and mount in thick cedarwood oil.

Mounting in gum dammar or balsam causes the sections to fade.

Medullary cells stain bright red to violet; cortical cells, blue to blue violet; red blood corpuscles, pink.

A good differentiation of adrenal medullary and cortical elements obtained by this method.

PANCREATIC ISLET CELL STAINS

Lane and Bensley Method

To distinguish between the A and B cells of the pancreatic islet tissue, Lane has devised the following technic:

1. Fix small fragments of pancreatic tissue for from 3 to 4 hours in the following fluid:

Potassium bichromate	-----	2.5 Gm.
Mercuric chloride	-----	5 Gm.
Distilled water	-----	100 c.c.

The sodium sulfate of the original formula may be omitted.

2. Dehydrate, clear, and embed in paraffin in the usual manner.

3. Cut sections 3 to 5 microns thick.

4. Stain for 24 hours in Bensley's neutral gentian, which is prepared as follows:

Dissolve 1 Gm. gentian violet in 25 c.c. distilled water. Dissolve 1 Gm. orange G in 25 c.c. distilled water. Mix and shake. A precipitate is formed which is separated by filtration, washed once with distilled water, and dried. Finally, it is dissolved in 25 c.c. absolute alcohol. This is a stock solution. For staining, add a sufficient amount of it to 20 per cent alcohol to give a deep violet liquid.

5. Blot section in filter paper.

6. Dehydrate and partly differentiate by a few seconds' treatment with absolute alcohol.

7. Blot rapidly.

8. Flood the slide with oil of cloves and control differentiation under microscope until acinus cells show purple zymogen granules and their ground cytoplasm is brownish yellow.

9. Clear sections in pure xylol.

10. Mount in gum dammar.

A cells: cytoplasm barely stained, nuclei pale purple.

B cells: cytoplasmic granules a brilliant purple, nuclei light purple.

Acinus cells: zymogen granules purple, ground cytoplasm yellow.

No acetic acid should be added to the bichromate solution as it dissolves the granules of islet cells.

Bensley's Acid Fuchsin Methyl Green Stain

Fix tissues for 24 hours in the following solution:

Osmic acid, 4 per cent aqueous solution	2 c.c.
Potassium bichromate, 2.5 per cent aqueous solution	8 c.c.
Glacial acetic acid	1 drop

Embed in paraffin in usual manner and cut sections 5 microns thick. Zenker's-fixed tissues can also be stained by this method.

1. Free sections from paraffin by toluol, then through absolute alcohol to water.

2. Treat with 1 per cent aqueous solution of potassium permanganate for 1 minute.

3. Treat with 5 per cent oxalic acid for 1 minute.

4. Wash thoroughly in water.

5. Stain for 5 minutes in aniline acid fuchsin solution previously warmed to 60° C.

The solution:

Acid fuchsin	2 Gm.
Aniline water, 5 per cent	10 c.c.

6. Wash thoroughly in water.

7. Dip for a few seconds in 1 per cent aqueous solution of methyl green.

8. Wash in water.

9. Dehydrate rapidly in absolute alcohol.

10. Clear in toluol and mount in gum dammar.

Acinus cells stain green with green nuclei; zymogen granules, red; basal filaments and mitochondria, red; granules of A cells, deep red; granules of B cells, green.

Goodpasture's Acid Polychrome Methylene Blue Method

For the differentiation of A and B cells of islet tissue:

1. Fix thin pieces of fresh pancreas for 24 hours in:

Formaldehyde, neutral	10 c.c.
Zenker's fluid without acetic acid	90 c.c.

2. Wash under running water overnight.

3. Dehydrate in graded alcohols and embed in paraffin. Cut very thin sections, 3 to 5 microns. Dissolve paraffin in xylol, wash in alcohol, then in water.

4. Treat in 1 per cent aqueous solution of potassium permanganate for 1 minute.

5. Treat in 5 per cent aqueous solution of oxalic acid for 1 minute.

6. Wash thoroughly in tap water.

7. Stain in aqueous solution containing 1 per cent eosin and 1 per cent potassium bichromate for from 1 to 5 minutes.

8. Wash rapidly in tap water.

9. Stain in acid polychrome methylene blue for from 1 to 5 minutes. See formula on page 129.

10. Wash rapidly in tap water.

11. Differentiate and dehydrate rapidly in 95 per cent alcohol and then in absolute alcohol.

12. Clear in xylol and mount in gum dammar.

Zymogen granules stain deep purple. Cytoplasm is light blue; nuclei, light purple; alpha granules, bright red; beta granules, pink blue.

Plasma Cell Stain (Unna)

Plasma cells supposedly arise from lymphocytes. They are abundant in subacute and chronic infectious diseases. The following technic of Unna demonstrates the plasma cells clearly:

1. Fix tissues in absolute alcohol. Fair results are obtained from tissues fixed in mercuric chloride, mercuric chloride-formol, or formaldehyde.

2. Make paraffin or celloidin sections in the usual manner.

3. Stain for from 20 to 40 minutes in Unna's pyronine methy green solution:

Methyl green -----	0.15 Gm.
Pyronine -----	0.25 Gm.
Alcohol -----	2.5 c.c.
Glycerin -----	20 c.c.
Carbolic acid water, 0.5 per cent -----	100 c.c.

4. Wash in tap water.

5. Blot in filter paper.

6. Dehydrate very rapidly in absolute alcohol.

7. Clear in xylol.

8. Mount in gum dammar.

Cytoplasm of plasma cells stains red; nuclei, bluish green. Lymphocytes also stain bluish green, but are easily distinguished morphologically from nuclei.

Mast Cell Stain

(Unna)

Fix tissues in absolute alcohol, and make thin paraffin or celloidin sections.

1. Stain in Unna's polychrome methylene blue for 2 hours in paraffin oven or overnight at room temperature.

2. Wash in distilled water.

3. Dehydrate in 2 changes of 95 per cent ethyl alcohol and blot.

4. Clear in oil origanum for 5 minutes.

5. Treat with neutral xylol.

6. Mount in gum dammar.

Mast-cell granules stain red; nuclei, blue.

MITOTIC FIGURE STAIN

Mitotic figures are brought out with sharpness when tissue is perfectly fresh; that is, immediately after removal from a living animal or human body. The details of mitotic figures are not so perfect on tissues removed at autopsies.

Tissue should be cut into very thin slices, not over 2 or 3 mm. thick, and fixed in a suitable fluid. Alcohol and Zenker's fluid are preferable.

Staining with alum hematoxylin or safranine gives satisfactory results, but phosphotungstic acid hematoxylin is highly recommended.

Mallory's Phosphotungstic Acid Hematoxylin

1. Fix tissues in Zenker's fluid for from 16 to 24 hours.
 2. Wash under running water overnight.
 3. Dehydrate in graded alcohols, beginning with 70 per cent, and finally in absolute alcohol.
 4. Embed in paraffin or celloidin, and cut sections.
 5. Treat with Lugol's iodine solution for from 5 to 10 minutes remove mercury precipitate.
 6. Wash in 2 changes of 95 per cent alcohol to remove every trace iodine.
 7. Wash in distilled water.
 8. Treat in 0.25 per cent aqueous solution of potassium permanganate for from 5 to 10 minutes.
 9. Wash in tap water.
 10. Treat in a 5 per cent aqueous solution of oxalic acid 5 minutes.
 11. Wash thoroughly in several changes of tap water.
 12. Stain in phosphotungstic acid hematoxylin for 24 hours.
 13. Transfer directly to 95 per cent alcohol, follow with absolute alcohol, and dehydrate quickly.
 14. Clear in xylol (filter paper blotting method for celloidin sections).
 15. Mount in gum dammar.
- Mitotic figures stain blue, as do the neuroglia, fibroglia, myoglia, and fibrin.

OXIDASE GRANULE STAIN

The following method is useful for differentiating myelocytes from the lymphocyte series; the myelocytes give a positive reaction, while the lymphocytes give a negative one.

Graham's Alpha-Naphthol-Pyronine Method

The material must be formol fixed, and freshly cut frozen sections must be used. After standing for from 24 to 48 hours in water, the granules may fail to react.

1. Stain sections lightly in alum hematoxylin (Harris). The solution must not be too acid.

2. Wash in water, then for about 5 minutes in a saturated aqueous solution of lithium carbonate. Return to water for a few minutes.

3. Stain for 10 minutes in alpha-naphthol-pyronine staining solution, made as follows:

Alpha-naphthol (Merck's "recrystallized" or Merck's "reagent") -----	1	Gm.
Alcohol, 40 per cent -----	100	c.c.
Hydrogen peroxide -----	0.2	c.c.

To 2 c.c. of this solution add immediately before use 1 drop of 2 per cent aqueous solution of pyronine.

4. Wash in water, then place for from 15 to 20 minutes in a saturated aqueous solution of lithium carbonate. Wash thoroughly in several changes of water.

5. Differentiate and dehydrate in 80 per cent, follow by 95 per cent alcohol. Transfer to a slide, and clear with xylol by the blotting method.

6. Mount in gum dammar.

The granules stain intensely red; nuclei, greenish-blue to blue.

Method of Examining Bone Marrow

The examination of bone marrow as either biopsy or autopsy material is a valuable adjunct to our diagnostic methods. The making of satisfactory preparations for microscopic study is rather difficult. The following method has given us satisfactory and constant results:

Place marrow in a small quantity of human blood serum and agitate for a few moments, then centrifuge for about 10 minutes at a moderate speed. Any fat that is in the marrow will rise to the surface and can be removed without difficulty. The supernatant serum is poured off, and any bone fragments that may be present in the marrow are removed.

The remaining sediment is mixed with 1 or 2 drops of serum and spread on ordinary glass slides in a manner similar to blood smears. These are fanned and dried rapidly, and are stained with a combination of Wright's and Giemsa's stain.

The resulting cellular detail in fresh material is excellent. Material from autopsy, where the body has been kept between 24 and 48 hours after death, is not so satisfactory.

CYTOLOGICAL DIAGNOSIS OF MALIGNANCY

For illustrations and more details on the so-called Papanicolaou method of diagnosis of malignancy, the reader is referred to two monographs: one entitled, "The Diagnosis of Uterine Cancer by the Vaginal Smear," by George N. Papanicolaou and Herbert F. Traut,* the other entitled, "The Epithelia of Woman's Reproductive Organs," by George N. Papanicolaou, Herbert F. Traut, and Andrew A. Marchetti.† Both of these books are remarkably well illustrated by drawings in color and photomicrographs. The artwork was made by Mr. Hashime Muryama. The original work was carried out entirely at the Cornell Medical School in New York City by Papanicolaou and Traut. Their work was performed with the cooperation of many members of the medical and surgical staff of Cornell University Medical School. This included thoracic surgeons, surgeons for gastroenterology, urologists, and teachers of obstetrics and gynecology. Another excellent manual is that by the Staff of Vincent Memorial Hospital of Boston, Mass., entitled, "The Cytologic Diagnosis of Cancer."‡ The illustrations in the book are amazingly clear and complete. The text is likewise very good.

Dr. George Papanicolaou has been a student of vaginal smears for many years. He began his work with a number of studies of smears of guinea pigs, for the purpose of studying the menstrual cycle in these animals. His first work along this line was published in 1917, together with C. R. Stockard. A later communication by him and Schorr appeared in May, 1936: this was a communication on the action of ovarian follicular hormones as indicated by vaginal smears. While carrying out this work he noticed pathological findings in the vaginal smears. Therefore, with H. F. Traut, formerly head of the Department of Obstetrics and Gynecology of the Cornell Medical College of the New York Hospital, facts were developed relative to the diagnosis of uterine cancer by vaginal smears. As indicated, this work has been applied not only to the diagnosis of

*Commonwealth Fund, New York, 1943.

†Commonwealth Fund, 1948.

‡W. B. Saunders Co., Philadelphia.

cancer of the cervix and uterus, but also to the diagnosis of pulmonary carcinoma, prostatic malignancy, cancer of the stomach, and even cancer of the rectum. In carrying out this work, the originators of the method had to prove its validity, and the problem of staining specimens properly. The validity of the method and the problem of staining have been established: it is now a question of the education of the profession and public. In making cytological diagnosis by this method, one must use a 15× ocular or wide-field ocular. With the 15× ocular and the low and high dry lens, we have the proper optical system. The oil immersion system is not necessary because the oil is difficult to remove and it is difficult to mark the point or cell when it is found. This should be done with a circle of ink or drop of ink. A spot on the surface of the cover glass and a ring beneath the cell will suffice.

The Vaginal Smear.—Smears must be taken from the fornix, from the surface of the cervix, and the endocervix. Cervical smears are made by use of a glass pipette with rounded end, so as to avoid trauma, attached to a rubber bulb. Material is drawn to the fornix and smeared with the end of the pipette over several glass slides which have been previously scrupulously cleaned. No lubricant should be used in the vagina for the purpose of insertion of instruments, etc., before the vaginal smear is made.

Having obtained the vaginal smear, we next prepare smears from the cervix by the surface biopsy method. This can be carried out by specially cut wooden applicators* with which the surface of the cervix can be swept and then the smears made on glass slides.

The final set of smears are made from the endocervix. These are prepared with a Beckton-Dickinson Abraham Laryngeal Canula No. 456 with a glass syringe attached. This is inserted into the canal and the material pulled out and smeared on glass slides.

Vaginal, surface biopsy, and endocervical smears, as soon as made and while still wet and properly marked, are dropped into a bottle containing equal parts of alcohol and ether for fixation. All smears may be left in the alcohol-ether mixture until they are stained and not dried at any time. If one wishes to stain at once, remove the slides after half hour in the alcohol-ether fixative and stain. If slides are to be mailed to a laboratory for staining, they are removed from the fixative after 1 hour and 2 or 3 drops of glycerin are

*Furnished by Clay-Adams & Co., New York City.

placed on the smear and a clean slide placed over it, the clip and label first having been removed. The 2 slides and label are pressed together with a rubber band. This is done with each type of smear.

Caution: Smears should not be made after a douche or bath.

Papanicolaou's Directions for Vaginal and Other Smears

Vaginal Smears.—Vaginal secretion is obtained by means of a glass pipette, spread on slides, and fixed immediately while wet in solution of equal parts 95 per cent alcohol and ether for a minimum of 5 minutes.

Endocervical and Endometrial Smears.—The secretion is obtained with a metal cannula (a laryngeal cannula may be used) and smears are prepared in the same way as vaginal smears.

Smears of Urine Sediment, Gastric and Bronchial Aspirates, Pleural and Peritoneal Fluids, etc.—The fluid is fixed immediately after aspiration in an equal amount of 95 per cent alcohol (50 c.c. of each are usually sufficient), centrifuged as soon as possible, at medium speed, for 30 minutes, and the supernatant fluid is removed. If smears cannot be prepared immediately, cover the sediment with absolute alcohol and place in refrigerator. Smears are prepared from sediment on slides which have been previously coated with a thin film of Mayer's albumen. The smear is spread with another slide as evenly as possible, and, when it starts to dry around the edge but is still moist in the center, the slide is fixed in alcohol-ether for a minimum of 1 hour.

Sputum Smears.—Sputum is collected directly in 70 per cent alcohol. It is spread on slides which have been coated with albumen. If there are hard masses they may be crushed slightly with another slide. The smear is fixed in alcohol and ether for a minimum of 1 hour.

Staining Method for All Smears.—After fixation in alcohol and ether, run the slides down through 80 per cent, 70 per cent, and 60 per cent alcohols to distilled water.

Stain in Harris hematoxylin (which has been prepared without acetic acid and diluted with an equal amount of distilled water) for 5 minutes.

Rinse in distilled water.

Dip in 0.5 per cent HCl (aqueous solution) 6 times.

Place in running tap water for 6 minutes.

Rinse in distilled water, 50 per cent, 70 per cent, 80 per cent, and 95 per cent alcohols.

Stain in OG6* for 1½ minutes.

Rinse in 95 per cent alcohol, 2 changes.

Stain in EA36† or EA65‡ for 1½ minutes.

Rinse in 95 per cent alcohol (No. 268), 3 changes; No. 267 absolute alcohol and xylol, equal amounts; xylol and mount with a cover slip.

BE SURE THAT SMEARS ARE NEVER ALLOWED TO DRY UP AT ANY TIME.

General Points on Diagnosis of Cancer by Vaginal Smears

In a lecture given by Dr. Papanicolaou before a class of fifty pathologists, of which one of the authors (R. B. H. G.) was a member, attention was called to the value of smears from vagina, surface biopsy, and endocervix. He regards endocervical smear as the most important, but in all cases, the vaginal smear should always be made. In the examination of vaginal smears, it was pointed out that cases of infection are easily made out by the microscopic appearance of bacteria as well as trichomonas. Relatively atypical cells are found in trichomas infection more so than in fungus affairs. The vaginal smear gives a very characteristic picture. The appearance of red cells may also give important information. Bleeding in connection with vaginal smear examinations may occur and some information can be obtained from the red cells. One can determine whether old, deteriorated red cells or fresh red cells are

*OG6:

Orange G (National Aniline and Chemical Co.) 0.5 per cent sol. in 95 per cent alcohol-----100 c. c.

Acid phosphotungstic (Merck)-----0.015 Gm.

†EA36:

Light green SF yellowish (National Aniline and Chemical Co.) 0.5 per cent sol. in 95 per cent alcohol ----- 45 c.c.

Bismarck brown (National Aniline and Chemical Co.) 0.5 per cent sol. in 95 per cent alcohol ----- 10 c.c.

Eosin yellowish (National Aniline and Chemical Co.) 0.5 per cent sol. in 95 per cent alcohol ----- 45 c.c.

Acid phosphotungstic (Merck) ----- 0.200 Gm.

Lithium carbonate, saturated aqueous solution ----- 1 drop.

The formulas of OG6 and EA36 are taken from the article, "A New Procedure for Staining Vaginal Smears," by George N. Papanicolaou, *Science* 95: 438 (Apr. 24), 1942.

‡EA65 is the same as EA36 with the difference that the Light Green is one-half strength (0.25 per cent instead of 0.5 per cent). It has the advantage of giving a lighter and more transparent staining which is desirable in smears containing much mucus. The differentiation between the acidophilic and basophilic cells is better with the EA36 (or EA50, which is more important in vaginal, endocervical, and endometrial smears. Therefore, EA36 or EA50 is preferable for vaginal, endocervical, and endometrial smears and EA65 for other types of smears, although any of these stains may be used for all types of smears.

These stains are furnished by the Gradwohl Laboratories, St. Louis, Mo.

resent. Red cells are not interfering factors in staining but sometimes favor this work.

Urine.—In regard to the technic of urine examinations, catheterized specimens are very important, especially in women. In the diagnosis of carcinoma of the prostate, catheterized specimens are referable. In the diagnosis of uterine carcinoma, the point must be emphasized that no douche should be taken before examination. The smear should be taken from the fornix of the vaginal cavity with a glass pipette and rubber bulb and rubbed evenly on the slide, after which it is immediately placed in equal parts of alcohol and ether before drying. There it should be left for at least 15 minutes; it may be left for as long as 2 days without any disturbance of the results.

Sputum.—This should be received directly into 70 per cent alcohol. The bottle should be half filled with this alcohol. It is not necessary to centrifuge this material. Take out a piece with a pair of forceps and rub directly with a wavy motion on a slide which has been rubbed with Mayer's albumen fixative. It may be necessary to crush the sputum smear with the end of the slide. Then the slides are fixed in equal parts of alcohol and ether for 1 hour.

The use of alcohol in the bottle for the sputum is for the purpose of prevention of bacterial contamination. It also better shows the cellular structure. The disadvantage of alcohol fixation is that we do not get as many cells as if we use the centrifuge method without alcohol, but its advantages outweigh this advantage. It is a disadvantage and an advantage at the same time. By this method you see certain patterns and clusters of cells. The smear has a pattern to be looked for. The alcohol fixation gives you a pattern by separating the cells.

Gastric Contents.—The examination of smears of gastric contents for the purpose of finding cancer cells presents difficulties not found in search for such cells in sputum or vaginal secretions. Possibly, the most important research on this subject has been made by Rosenthal and Traut,* who call attention to the presence of mucus in gastric aspirates as a serious obstacle to the staining and finding of cancer cells; mucus actually reduces the number of exfoliated cells. They found that the common proteolytic enzyme, papain,

*Rosenthal M., and Traut, H. F.: *Cancer* 4: No. 1., Jan., 1951.

activated to cysteine hydrochloride, causes rapid mucolysis. The cells are intact long after the mucus is completely liquified; but because of the absence of mucus, they failed to stick to the slides to which they were applied. Adhesiveness was renewed by mixing a small amount of commercial gastric mucus with the centrifugate. This can be done by adding a pinch (0.2 Gm.) to the digest just before centrifugation, with brief stirring. It was found that best results could be obtained by direct lavage of the stomach with the papain solution after the fasting specimen was removed. Many of the cells found in the fasting stomach contents show a considerable degeneration of the cells presumably because of the length of time since they had been exfoliated. Confusion is also added because of the presence of food particles. Finally, experience with surgical and autopsy stomach sections indicates the rich cellular content of the "mucous barrier" that lines the mucosa. No damage is done by papain lavage.

Method

The lavage solution is made up fresh. Whether the solution is stable for a longer period of time is not yet determined. An excess of papain (3 level teaspoonfuls) and approximately 3 Gm. of the activator cysteine hydrochloride are ground in a mortar and added to 500 c.c. of buffer solution, briefly stirred, and filtered. Coarse ("gray crepe") paper is preferable to the finer grades, which tend to clog. (The buffer used is an isotonic one, consisting of 350 Gm. of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and 0.4 moles [e.g., 400 c.c. of 1 N] of HCl added to 5 gal. of distilled H_2O , giving a pH of about 7.2 to 7.4.) It has been found unnecessary to adjust the proportions further to attain the aforesaid pH value exactly. Indeed, it may not be necessary to use buffer at all, since papain is active over a wide pH range. However, the use of buffer was decided upon to protect the cells, liberated from mucus, from the action of free hydrochloric acid. Whether this action is a deterrent during the short time between removal of the instillate and fixation of the cells will be investigated in the future.

The filtrate is instilled into the stomach through a Levine tube. A 3 oz. Asepto syringe is most convenient for instillation and removal. The instillate is removed after ten minutes, at which time mucolysis is almost always complete. If a few tiny mucus shreds are still present, they may be removed by pouring the digest through several layers of gauze.

Usually about 450 c.c. of solution are recovered without difficulty. Occasionally, less is recovered and several times none was recoverable. No disturbance has been observed in any case.

The volume of 500 c.c. was selected in order to ensure contact with the entire stomach surface. In ordinary barium fluoroscopy, 50 c.c. of barium suspension usually fills the stomach without distention.

After removal of the turbid suspension, no evidence of cell digestion is apparent for an hour. Centrifugation for five to ten minutes at moderate speeds of the total lavage return, or of a well-mixed aliquot, yields an abundant and purely cellular sediment, usually to 4 c.c.

According to Rosenthal and Traut, no patients known to have carcinoma, or with a clinical diagnosis of carcinoma, have been missed by this method of examination.

The use of Mayer's fixative on the slide is to make the sediment stick to the slide. A single drop of the fixative is placed on a slide and the finger is used to spread it. After the sediment is placed upon this fixative, just when it begins to dry around the edges, the slide should be dropped into alcohol and ether. If you place it into the alcohol and ether too quickly, the material will wash off. If he waits too long, the same thing will occur. The longer one keeps the slides in alcohol and ether, the better the cells will stick. If it is inexpedient to stain this material immediately, pour off the supernatant fluid and cover with absolute alcohol and put it in the rebox. Centrifuge for 30 minutes. Do not rinse too vigorously to avoid detaching some of this material from the slide. Be careful in drying the slide not to contaminate it with material from another case. Touch it lightly with the paper for drying.

Criteria of Malignancy.—Smears for malignancy were formerly reported as *negative* or *positive*. Some were considered "suspicious." Later, a new method of classification was developed. In reference to urine, there were cells which were called atypical. Then Papanicolaou developed classifications I to V.

Class I is reported negative with the inference that there are no cells present which suggested malignancy. Atypical cells occur in chronic cervicitis. Many of these cells are vacuolated but they are not malignant. Nuclei of such cells are normal.

Class II or atypical cells such as are seen in chronic cervicitis. Sometimes there are clusters of cells and endometrial cells and

histocytes. These cells have no nuclear aberrations. In other words, Classes I and II are negative.

Class III. In this class, there appear to be no malignant cells but yet these cells may later be classified as positive. Fifteen per cent of reports are called Class III. They are "suspicious" cases. For all practical purposes, 50 per cent of these cases (Class III) later upon re-examination turn out to be malignant.

Class IV comprises individual malignant cells with overwhelming evidence of malignancy in the cellular structures.

Class V are definitely malignant cells. According to Papanicolaou, one may take the responsibility for a major operation on this diagnosis. A biopsy, however, should be made, but even a biopsy may be negative in these cases. These are often cases without symptoms. It is not ordinarily a method of final diagnosis but still Papanicolaou assumes responsibility for an operation. He has found these cells in early bronchogenic carcinoma.

Respecting Class IV, occasional mistakes in sputum examinations have been made; later it was found that the cells which were mistaken for malignant cells came from cases of bronchiectasis, where there are clusters of cells simulating malignant cells but were not malignant. It is possible with our present knowledge of these cells to make a diagnosis of bronchiectasis from these atypical cells; they are mucoid cells arranged in clusters.

Respecting diagnosis of urogenital malignancy, with examination of more specimens, especially for use in catheterized specimens, there should be less false negatives.

In gynecological statistics, it is difficult to evaluate all the facts. In Papanicolaou's early work, the specimens were not well handled. The use of endocervical smears has increased the accuracy. In the beginning, when only vaginal smears were examined, results were not as accurate. With present methods, less than 10 per cent of the cases are missed. Endocervical smears are the most important material to use.

How many carcinomas are missed by biopsy? The answer is, many. It is better, of course, to use both methods—smears and biopsy—to make a report of the highest accuracy. The standing of the method will not be injured by false negatives, because they can always be followed up by biopsies and curettements.

In order to get the highest type of reliable results, Papanicolaou emphasizes the importance of well-prepared and well-stained speci-

nens. It is impossible to do this work without following the proper technique. These methods should appeal to the pathologist; there should be no quarrel between the pathologist and cytologist over this question.

Regarding the criteria of malignancy: nuclear enlargement is considered to be the most important of all. The nuclear size is disproportionate to the size of the cell. There is a discrepancy between the size of the cell and the nucleus. There is anisokaryosis. There may be free or extruded nuclei. There are certain cells found in specimens from ureter catheterization which are binucleated.* They come from the pelvis of the kidney. They also have oversized nuclei. They are transitional cells and must not be confused with malignant cells. You will find these irregular-appearing cells in the urine from ureteral catheterization. Sometimes only actual drawings of each specimen will show the difference between various types of irregular cells. Also one may find ciliated cells with large nuclei which are not malignant. The size of the nucleus is a very important criterion.

The structure of the nucleus is very important, likewise the chromatin in the nucleus. They are prophaseic. Nuclei have prominent chromatin. Hyperchromasia may occur, which may be due not to increase of the chromatin but to degeneration of chromatin. Chromatin is prominent. There are mitoses normally. Mitoses are rare in exfoliated cells. It is not because such cells are superficial. No mitoses are found except in single cells. Mitotic figures are suggestive but not absolutely positive. As for histiocytes, these are phagocytic cells. They may show mitotic figures. In regard to fragmentation of nuclei, binucleation is not fragmentation. There is irregularity in the breaking-down of nuclear structures. Overstaining a slide may convince you of hyperchromasia. This might confuse one in making a diagnosis of carcinoma.

Basophilia and acidophilia are seen in vaginal smears. The acidophilic cells stain like cornified cells. Such cells are pink or orange. In adenocarcinoma, one cannot depend on one or two slides, but one may do so in epidermoid carcinoma.

Another criterion is vacuolization which is more common in adenocarcinoma. It may mean only metaplasia, not necessarily malignancy.

*One of the authors (R. B. H. G.) has encountered two such instances, where the kidney pelvic cells closely simulated cancer cells.

nancy. It is particularly true of endometrial cells which may be mistaken for malignancy. Vacuolization is found in adenocarcinoma.

Cytoplasmic Inclusions.—These are small cells. How do we interpret them? Is this a phagocytosis? It is not phagocytosis. Inclusion bodies are important in adenocarcinoma. One finds them in chronic infections. In adenocarcinoma these cells are not degenerative. In adenocarcinoma large cells are present; they are “mucoid,” which, by exfoliation, tend to become round. The nucleus becomes basic and excentric. In adenocarcinoma they appear in clusters but without histological qualities. In endometritis one does not find such cells. Fragments of endometrium coming from the tunica propria may be found. Metaplasia with vacuolization of the endometrial cells may occur. Such cells are telescoped into each other. Such cells may be found in malignancy, but not necessarily.

There are “aberrant” forms of cells representing gigantism of cells. The presence of degenerated cells is a very important criterion. It is of particular importance in adenocarcinoma of the endometrium. These are small cells with the nuclei breaking down. There is necrosis and degeneration in such cells. There are also many degenerated cells in cases of chronic infections. Criteria in general are histological as well as cytological.

Presence of nucleoli is a good criterion in certain types of carcinoma. Nucleoli are sometimes present, sometimes not. In bronchogenic carcinoma nucleoli are an outstanding symptom. You will find large nucleoli in single cells; for instance, in transitional cells.

Cytological Criteria.—

1. Crowding. This is better found in smears than in sections.
2. Irregularity in the pattern and in the size of the nucleus.
3. Around the cytoplasm there is a ring, which is an indication of a normal cell.
4. Another criterion is the loss of individuality of cells. In carcinoma there is loss of nuclear outline, or the cells are extremely indefinite. There is anisokaryosis within a group.

Clusters of cells press into each other.

Blood.—In sputum, clusters of cells are found in positive cases of bronchogenic carcinoma, due to breaking down of the lymph nodes.

The presence of polymorphonuclears does not give much information. Adenocarcinomas are frequently associated with infections in which are found many "polys."

Plasma cells indicate chronic infection. Histocytes in size look like monocytes. Lymphocytes are easily recognized. Eosinophiles are found with eosin stains but not with the Papanicolaou method.

Bleeding.—Very rarely do you find bleeding in malignancy. There may be normal menstruation, menorrhagia, or amenorrhea. You can produce bleeding artificially in surgical or biopsy preparation. Polymorphonuclear cells are phagocytic. They may attack spermatozoa but not red cells. In abortion, the polymorphonuclear cells attack red cells. The type of bleeding may indicate pathology.

Regarding the specific picture of threatened abortion from the cellular smear examinations, the effect of stimulation of estrogenic function is the presence of more paranuclear granules. But these granules are found in other conditions. Another criterion is the size. In the menopause or amenorrhea we may find such cells, but they are very small. They do not stain so well. The granulations stain like chromatin.

As for the picture in threatened abortion, the most important thing is bleeding. There is no bleeding in normal pregnancy. The bleeding is the first symptom of threatened abortion. Therefore, the presence of blood in the smear is very important. Another thing is the presence of excessive free mucus in the smears and the relatively large number of acidophilic cells.

Staining Method.—There is no specific staining method for cancer cells. When the work was first started years ago, hematoxylin and eosin were used. Later, it was found that they did not give good distinction between basophilic and acidophilic cells. It was found that water blue was better and it was used for many years. Later, workers tried to prepare a better stain, preferably an alcoholic stain, which gave more transparent staining. It was realized that light staining was necessary. It was necessary to get a light pink or orange stain. It was then that orange G was first used. Hematoxylin and orange G alone do not give enough differentiation. Then the EA series was started. It is held that the yellow stain is the basis for all the stains. Hematoxylin and eosin do not give enough differentiation between the acidophilic and basophilic elements. This is exceedingly important in sputum where such a distinction is necessary. It is not important in the urine. Hematoxylin is too dark in its staining effect. If there is bleeding and much mucus,

the staining is also too dark. You must have good transparency in all this work. The present staining method was published in 1940.

The staining is not specific. The important point in all this staining is to have good nuclear staining. It is not probable that we can have a single stain for both nucleus and cytoplasm. In 1941 Schoor published a single stain which is excellent in distinguishing between acidophilic and basophilic qualities. Papanicolaou does not believe that any single stain can be used for any cancer diagnosis. Also, single stains fail with blood and mucus. With the new method of staining we can detect the criteria of ovulation because acidophilic cells do not disappear or diminish but remain in large numbers for a few days after ovulation. At times they are present before ovulation.

At the time of ovulation, one may seek keratinization or cornification. There is no specific cellular ovulation appearance. You can detect the number of cells as the direct result of ovulation. This limits the possibilities of this method. You cannot, from the smear, tell whether it is ovulatory or syphilitic. It can be told at times with typical cells but you must not expect to do this always.

This method may be used as a substitute for frozen sections. Staining methods for this work must give nuclear staining with good differentiation.

Attention is called to the need of looking for ciliated cells. Look for ciliated cells in staining smears for hyperplasia. It is important to have a stain by which you can detect glycogen and ciliated cells.

Menopause.—In menopause there may be many different types of cells. There may be a secretion of estrogen for a long time. It depends upon the level. The ovarian follicles may be active a long time. This may account for what we call postmenstrual cycles. There may be ovulation after menopause. In other words, there is a transitional period which is reflected in the vaginal smear. One may find many acidophilic cells. There are various levels dependent upon follicular activity. It all depends upon the level of the secretion of estrogens. There may be menopause levels just as in menorrhoeas.

“Crowded” basophilic cells are found in many levels, also leucocytes, because these cases are associated with severe cervical infection.

In low levels, good results are not obtainable from gonadal preparations.

Local Infections.—Local infections and trichomonas infections can use the picture. They produce acidophilic cells.

Cells show granules which are broken off from the nucleus. Such cells are found in normal urine where estrogenic drugs have been injected or taken. These granules are found in the identification of basophilic cells which are the results of estrogenic therapy.

Pregnancy.—In pregnancy there are changes in the epithelium of the bladder, or transitional cells because of the glycogenic secretion. These cells take the same form as those of the vagina, namely, avicular or boat-shaped.

In the vagina you may find such cells without estrogen but in male urines it indicates estrogenic therapy.

Effect of Radiation on Cells.—There are certain changes in cells producing a necrosis. The cells do not stain very well. The nucleus stains like the cytoplasm. They do not take the stain. The nuclear outlines disappear.

Polymorphonuclears enter the cell, which indicates the effect on the cells by x-ray. Leucocytes are in the cytoplasm, near the nucleus. It is a good idea to make a series of smears after radiation.

Value of Urine Examinations by Papanicolaou Method.—In a contribution by Gradwohl and Hall,* presented before the Southwest Branch of the American Urological Association, the value of urine examination by Papanicolaou's methods was discussed. It gives information on bladder, prostate, and kidney pathology. Cancer cells frequently enter the urine from the prostate when one would not expect this to occur. It may help in the diagnosis between benign and malignant papilloma of the bladder.

Method of Staining Effusions for the Discovery of Cancer Cells in Neoplastic Exudates

(Shu Chu Shen and Homburger)

Shu Chu Shen and F. Homburger† report a new and simple procedure for the preservation of cellular structure and the rapid and reliable detection of cancer cells in exudates by means of a modified Wright stain.

Method.—The freshly drawn aliquot (about 200 ml.) of effusion fluid is defibrinated in an Erlenmeyer flask containing forty glass

*Gradwohl, R. B. H., and Hall, A., Jr.: Urol. & Cutan. Rev. 53: 257-262, 1949.

†Shu Chu Shen and Homburger, F.: Cancer, Jan., 1950. Published by Paul B. Hoeber, Inc.

beads. About 20 ml. of the defibrinated fluid is placed in each of two test tubes ($5/8 \times 5\frac{3}{4}$ inches) and centrifuged at 3,000 r.p.m. The supernatant is pipetted off and discarded. The precipitate is then resuspended by gentle agitation in 2 to 3 ml. of homologous serum or serum previously adsorbed by red cells of groups A and B. This suspension is allowed to stand at room temperature for 10 minutes; it is then transferred to a smaller test tube ($3/8 \times 4$ inches) and spun once more for 5 to 10 minutes at 3,000 r.p.m. The supernatant is again withdrawn and discarded, except for a small amount of serum that is allowed to remain. Using a capillary glass pipette, such as is employed to fill Wintrobe hematocrit tubes, a drop of the precipitate is taken from the top layer of the sediment and placed on clean cover slips for the preparation of smears by the two-cover-slip method of Ehrlich. After drying, the smears (preferably 3 to 6) are covered with Wright stain, to which an equal amount of phosphate buffer of pH 6.4 is added after 5 minutes. This is left for another 5 minutes. The smears are then washed, dried, and mounted on a slide for microscopic study. The cells are frequently present in great number but occasionally they are so scarce that several slides will have to be examined.

These writers claim that the appearance of neoplastic cells stained by the procedure above outlined is characteristic and easily distinguishable from that of desquamated mesothelial cells occasionally found in transudates. Descriptions of these cells may be found in the legends to the illustrations in the original article. The procedure gave no false positive findings in a limited number of cases and fewer false negative results than were obtained by Papanicolaou's technic.

PART IX

BOTANICAL MICROTECHNIC

E. D. Woodhouse, Ph.D.*

In presenting a single chapter on botanical microtechnic in a complete text, no effort is made to be comprehensive or to present original material, but rather to offer the beginning student adequate guidance in the best procedures, many of which are here collected under one cover for the first time.

In comparing botanical with zoological microtechnic, consideration must be given to the difference in the nature of the materials being handled. With the exception of bone, most animal tissues are soft and require hardening. Most plant tissues, on the other hand, are fairly rigid, so that procedures which soften rather than harden are preferred. At the other extreme are filamentous algae and fungi, which are more fragile than most animal tissues but to which ordinary procedures are not applicable.

Another difference is that many plant tissues are filled with air spaces, so that it is necessary to subject the material to a partial vacuum after placing it in the fixing fluid in order to insure the thorough penetration of the latter. This is accomplished in the laboratory through the use of a rubber-stoppered vessel into which the small shell vials containing the fragments of plant material in the fixing fluid are set. Two pieces of glass tubing extend through the stopper; one is connected to a suction pump, and the other serves as a release valve, which is closed with a finger during evacuation. Special equipment is necessary to accomplish evacuation outside the laboratory.

The general principles involved in procedures are the same in both fields. For proper fixation of tissues, small portions only should be used. Additional formulas of specific application are given. The equipment is essentially the same in both fields. Since botanical technic does not always involve the making of sections, consideration of this phase of the subject is deferred and com-

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bined with a discussion of other methods, preceded by additional information on staining solutions applicable to plant tissues. Clearing and mounting reagents are already completely covered under their respective headings, with the exception of two additional mounting media.

Fixing Reagents

Formalin Acetic Alcohol (FAA)

Alcohol, 50 per cent	900 c.c.
Formaldehyde, 40 per cent	50 c.c.
Glycerin	25 c.c.
Acetic acid, glacial	25 c.c.

Formalin acetic alcohol is the most generally applicable reagent for use with plant tissues. Material can be transferred directly from this reagent to 50 per cent or 70 per cent alcohol for dehydration, or a solution of stain for mounting in glycerin jelly.

When whole preservation is desired, the addition of 10 per cent copper acetate or chloride to this formula will simulate the normal green color of plants, which is further improved by 1.5 per cent uranum nitrate.

Navashin's Fluid

(Belling's Modification)

Navashin's fluid is excellent for fixing chromosomes in root tips, pollen grains, etc.

Solution A:

Chromic acid crystals	5 Gm.
Acetic acid, glacial	50 c.c.
Distilled water	320 c.c.

Solution B:

Formaldehyde, commercial	200 c.c.
Distilled water	175 c.c.

Mix A and B in the ratio of 1:1 immediately before using.

Fix 18 hours or longer.

Wash in running water.

Flemming's solution (see page 22) is also suitable for this purpose.

Petrunkewitsch's Fluid

Petrunkewitsch's fixing solution is especially valuable for meiosis in plant cells.

Alcohol, 40 per cent	250 c.c.
Acetic acid, glacial	45 c.c.
Nitric acid, concentrated	5 c.c.
Mercuric chloride	to saturation

Make up only as required.

Fix 12 to 18 hours.

Wash thoroughly in 70 per cent alcohol.

After embedding, remove mercury salts from sections in the following solution:

Alcohol, 50 per cent	100 c.c.
Iodine	1 Gm.
Potassium iodide	1 Gm.

Remove the iodine with 0.2 per cent aqueous solution of sodium thiosulfate (hypo).

Chamberlain's Solution

Chamberlain's formula is particularly good for filamentous algae and fungi where it is desired to avoid plasmolysis.

Chromic acid	1 Gm.
Acetic acid, glacial	3 c.c.
Osmic acid, 1 per cent	1 c.c.

With 2 c.c. osmic acid, it is recommended for mitosis in root tips.

Osmic Acid Vapor

For fixing the cilia on zoospores and antherozoids, invert a slide with a drop of culture material for 30 seconds over a bottle containing osmium tetroxide crystals or a 1 per cent or 2 per cent solution in dust-free distilled water. A trace of chromic acid or potassium permanganate may be added to prevent the reduction of osmic acid.

Warning! Osmic acid vapors are poisonous.

Mercuric Chloride, Acetic Acid, and Formalin

Mercuric chloride -----	4 Gm.
Acetic acid, glacial -----	5 c.c.
Formaldehyde, 40 per cent -----	5 c.c.
Water or alcohol, 50 per cent -----	100 c.c.

Use water where dehydration is not necessary.

This fixing fluid is used to preserve filamentous and unicellular forms and delicate structures including cilia.

Handle material in this solution with wooden forceps.

Wash out the mercuric chloride in water or alcohol; any residue will decolorize iodine solution.

Desilicification

Soak the material in hydrofluoric acid to dissolve out the silica. Hydrofluoric acid must be used in a thoroughly paraffined container.

To Soften Wood

Boil in glycerin.

Equipment

Identical equipment is used in either zoological or botanical microtechnic, emphasis on the individual items used varying with the procedures followed.

A sliding microtome of simpler construction than the freezing microtome is adequate for sectioning plant stems. The plane concave knife is used for this purpose.

Whereas Krajian prefers the paraffin microtome manufactured by Bausch & Lomb Optical Company, I prefer the one manufactured by the Spencer Lens Company. No doubt those of foreign manufacturers also have their adherents.

An additional major item of equipment of great convenience is a Ransom thermostatically controlled electric slide warmer.

Other minor items of equipment supplementing those listed (see pages 26 to 44) are:

1. Syracuse watch glasses.
2. Cover slip forceps.
3. Diffenbach's straight cross action clamp for holding slides.
4. Straight edged scalpel for trimming paraffin blocks.



Fig. 115.—Simplified clinical microtome for botanical work. (Courtesy Bausch & Lomb Optical Co.)

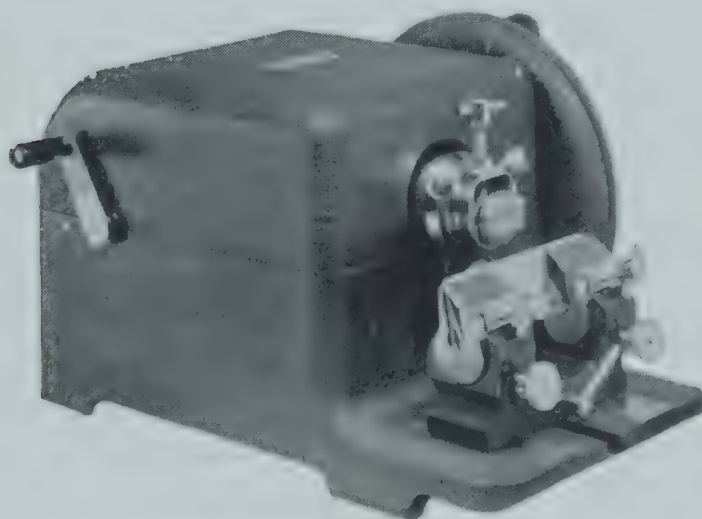


Fig. 116.—Paraffin microtome (Spencer).

5. Old scalpel or knife for mounting blocks.
6. Shell vials.
7. Aluminum foil for embedding boxes.

Staining Solutions

Without considering the relative merits of the physical and chemical theories of staining, for practical purposes stains may be divided into basic dyes which stain acid structures, and acid dyes which stain structures of alkaline reaction, making possible counterstaining by the use of two successive and complementary dyes.

Basic dyes are held fast by lignified xylem, suberized cork, cutinized epidermis, or chitinized cell walls, and by chromosomes, centrosomes, and nucleoli.

Acid dyes are held fast by achromatic nuclear structures, plasma, cilia, cellulose cell walls, and starch.

Basic Dyes

Safranine. The best basic red dye. Counterstain with fast green or aniline blue.

Hematoxylin. (See page 123.) This is the best nuclear stain. Harris' or Heidenhain's formula preferred. The latter can also be prepared for immediate use by ripening with a trace of sodium bicarbonate.

Brazilin is a related dye.

Methyl green. Used with acid fuchsin (red) as a counterstain. Obviously, fast green could not be used as a counterstain.

Gentian violet. Used as a supplement to safranine, or even as a counterstain, the results depending upon which is applied first. Erythrosin is a proper counterstain for gentian violet.

Crystal violet. A related dye which can be used as an alternative in any procedure calling for gentian violet.

Magdala red. Used for algae with aniline blue as a counterstain.

Janus green B. Used for staining mitochondria.

Carmine. A natural red dye, varying in stability according to the manner of use.

Acid Dyes

Fast green. One of the most widely used counterstains, replacing others such as *Light green*.

Acid fuchsin. A red counterstain. Obviously cannot be used with safranine.

Orange G and Methyl orange (gold orange) are used as background plasma stains.

Eosin, Erythrosin, and Phloxine are related red dyes, tending to blue or yellow in shade according to the specific formula chosen.

Aniline blue is commonly used where a blue counterstain is desired.

Martius yellow and Malachite green are used to distinguish host tissues from fungus mycelium in the Pianese stain combination.

Nigrosine (induline). A blue-black dye used on algae and fungi, with phloxine as a supplementary stain.

Cyanine. A similar dye used as a counterstain for carmalum.

Preparation of Dye Solutions

Most of the aniline dyes are used in aqueous or 50 per cent alcoholic solution, with a concentration of 1 per cent; or in a 95 per cent alcohol solution mixed in equal parts with a saturated solution of aniline oil in water; or diluted from a stock solution of methyl cellosolve, described below.

The following counterstains are frequently used as a saturated solution in clove oil: fast green, orange G, aniline blue, and erythrosin. The addition of absolute alcohol to the clove oil increases the solubility of the dye, or better yet, use methyl cellosolve (ethylene glycol monomethyl ether), as recommended by Johansen. Make a saturated stock solution of dye in methyl cellosolve and add as needed to clove oil containing 1 part in 5 of absolute alcohol.

Clearing Reagents

See page 134 ff.

Mounting Reagents

See pages 136 and 138.

Venetian turpentine is the resin of *Larix europæa*. It is an excellent medium for filamentous algae and fungi, but requires a desiccator for concentration during infiltration, and may crystallize with age.

Hyrex is a neutral synthetic resin having a refractive index of 1.822, which is higher than that of glass; hence it is excellent for mounting the glass shells of diatoms.

Methods in Botanical Microtechnic

General Instructions Applicable to All Procedures

Transferring from solutions of one osmotic concentration to another should be performed gradually by stages and over a more or less extended period of time, varying with the size and delicateness of the material being handled. This applies to both material in bulk and to sections on slides. These stages can be attained either by adding new and by subtracting old portions of fluid in one dish, or by using a series of dishes of varying concentrations of reagent, such as those of the following percentages: pure water, then 5, 11, 18, 30, 50, 70, 85, 95, and 100 per cents. For finer work, use closer gradations than for coarse work, skipping some of the above steps if desired in the latter case. Keep a petroleum jelly-sealed cover on 100 per cent alcohol or mixtures made with 100 per cent alcohol.

It is possible to enter such a series at the proper point from another solution of like concentration, or to digress from such a series at any point in order to use a staining solution of a concentration the same as that at the point of digression, reentering the same series at the next step. It is also possible to go in either direction in such a series.

In any procedure the course should be planned with understanding, as in arranging a journey; one should not follow blindfolded a series of "rules of thumb."

Fixing. The fixing fluid should be applied to the material in such form and size that penetration and killing will be rapid and thorough, but the solution should not be so strong as to cause disintegration. Different kinds of fluids may produce entirely different results in the finished product. See page 271 regarding evacuation during fixation.

Washing. Aqueous fixing fluids should be washed out of the material in copious amounts of water or in running water. Alcoholic fixing fluids and picric acid should be washed out in 50 per cent alcohol, or alcohol of the same concentration as that used in the fixing fluid.

For marine materials, the fixing fluid should be made up with half sea water, and the washing should commence in sea water.

Staining is accomplished before, during, or after dehydration, according to the type of dye solvent used. Staining reactions should always be controlled by microscopic observation. Protect the stage of the microscope with a glass plate.

Differential Acidification. Treatment of tissues with an acid prior to staining with the basic dye tends to sharpen differentiation. Acids used in various procedures are: normal hydrochloric, 1 per cent acetic with 0.1 per cent sulfuric, and saturated solutions of tannic or picric acids. Wash out the excess acid before staining.

Staining in aqueous dyes follows the washing out of the fixative in the case of unmounted bulk material; go down an alcohol series to water if the washing fluid was alcohol. In the case of sections attached to slides, it is always necessary to go down through the series when using an aqueous stain. The use of an aqueous counterstain is possible by selecting a proper combination of dyes.

Dehydrating partially can be accomplished by concentrating in a dilute solution of glycerin.

Partial or complete dehydration is obtained by an alcohol series, ending in 100 per cent for complete dehydration. Ethyl alcohol is standard. Isopropyl alcohol is an accepted substitute where the former is unobtainable. Butyl alcohol is preferable preceding paraffin embedding.

Staining in alcoholic dyes follows washing in alcohol or digression from the alcohol series. Both staining and counterstaining can be performed at this point by washing in alcohol of the same concentration between the two dyes. Since the first dye is usually the basic, addition of a trace of hydrochloric acid or a larger amount of picric acid to the wash solution sharpens differentiation.

Counterstaining with dyes dissolved in clove oil follows the completion of dehydration, either before or after transferring the material or sections to xylol. If acid alcohol was used to obtain differentiation, a final wash of alcohol with a trace of ammonia should precede the 100 per cent alcohol.

Clearing is accomplished by transfer to xylol from the 100 per cent alcohol, either through clove oil or a 1:1 mixture of xylol and absolute alcohol; or to Venetian turpentine directly from 100 per cent alcohol. Glycerin itself forms the clearing agent where it is used for partial dehydration.

Mounting in glycerin jelly follows the use of glycerin for clearing, and in balsam follows the use of xylol. Venetian turpentine, when used for clearing, becomes the mounting reagent by concentration in a desiccator. The cover glass should be applied at one edge first.

resting the free end upon a needle or forceps, and lowered evenly and smoothly to exclude air bubbles by withdrawing the instrument.

The slides, in the case of balsam, should be dried on a hot plate or in an oven at about 50° C.

Mounting in Glycerin Jelly

The procedure of mounting in glycerin jelly is suitable for whole mounts of filamentous or other small forms, and sections, such as the freehand lengthwise sections of moss capsules.

Advantages:

1. Filamentous algae and fungi are usually sufficiently transparent to be studied without sectioning.
2. Fixing and staining may be omitted without complete loss of the natural color and without deterioration of most materials.
3. Complete dehydration, with the danger of plasmolysis of fine structures, is obviated, since glycerin is miscible with water.
4. The procedure described herewith obviates the use of a turntable and fragile shellac ring to seal the glycerin against desiccation. Slides prepared in the following manner have been in use for ten years:

Procedure:

1. Fix.
2. Wash.
3. Stain in aqueous eosin or hematoxylin.
4. Rinse in water with a trace of acetic acid.
5. Slowly add a small amount of glycerin to the material in a flat dish full of water and proceed with Step 6.

Alternative Procedure:

Start with Step 5, omitting Steps 1 to 4.

6. Cover with filter paper to exclude dust but permit evaporation.
 7. Concentrate by evaporation to a thick consistency.
- Mount in glycerin jelly on individual slides as follows:
8. Dissect or separate out a small fragment of material sufficient for one slide and transfer to the center of a 22 mm., No. 1 cover glass, held at the edge by cover glass forceps.
 9. Add a small particle of glycerin jelly.

10. Warm over an alcohol lamp or other flame in order to melt the jelly and drive out air bubbles, but not sufficiently to create additional bubbles by boiling.

11. Place an 18 mm., No. 2 cover glass on top of the melted jelly containing the specimen, centering it over the 22 mm. cover glass. The portion of glycerin jelly used should be sufficiently small that none of it extends beyond the edge of the 18 mm. cover glass.

12. Cool to solidify the jelly.

13. Invert into a large drop of balsam on a clean microscope slide. The balsam should be sufficient to completely fill the ring where the 22 mm. cover glass extends beyond the 18 mm. cover glass. In this manner a perfect seal is formed which is not subject to breakage, as in the case of a shellac ring extending above the surface of the cover glass.

14. Dry on a hot plate or in an oven at about 50° C.

Mounting in Venetian Turpentine

Advantages:

1. Does not require sealing.
2. Permits greater variety in staining.

Disadvantages:

1. Requires a desiccator.
2. May crystallize with age.

Procedure:

1. Fix.
2. Wash.
3. Stain in aqueous stains, such as hematoxylin or eosin.
4. Transfer to 10 per cent glycerin and concentrate as for mounting in glycerin jelly.
5. Wash in 95 per cent alcohol.
6. Counterstain at this point if desired.
7. Dehydrate in 100 per cent alcohol.
8. Decant the alcohol.
9. Set the dish in a desiccator. (An airtight container having a layer of dry soda lime or calcium chloride may be used.)
10. Add 10 per cent Venetian turpentine.
11. Concentrate in the desiccator to a thick consistency.
12. Mount the material in a drop of the Venetian turpentine and add a cover glass.

Galigher's Method of Mounting in Balsam

Galigher's method of slow infiltration with balsam overcomes the disadvantages of Venetian turpentine.

Procedure:

1. Fix.
2. Wash.
3. Stain in aqueous or alcoholic stain.
4. Dehydrate in alcohol.
5. Counterstain at this point if desired.
6. Complete dehydration in 100 per cent alcohol.
7. Transfer gradually to creosote-xylol solution (1:1 xylol and beechwood creosote).
8. Cover material with pure creosote-xylol solution to a depth of $\frac{1}{4}$ inch in a low dish.
9. Introduce a filter paper cone with the tip touching the xylol and the top resting upon the edge of the dish and held in place by a cover.
10. Add into the cone 5 drops of balsam twice daily to produce a medium syrup in from several days to 2 weeks.
11. Remove the cover and filter paper to evaporate to a thick syrup.
12. Mount on a slide and apply a cover glass.
13. Dry.

Johansen's Method of Mounting in Balsam

Johansen's method employs "Hygrobutol," a nearly universal solvent, which is a trade mixture of tertiary butyl alcohol and a hygroscopic reagent. It eliminates xylol from the series, and materials which do not readily plasmolize can be placed in it directly, eliminating the alcohol series. Where there is danger of plasmolysis, the glycerin jelly method still remains the simplest and most direct.

Procedure:

1. Fix.
2. Wash.
3. Stain, partially dehydrate, and counterstain, *or* partially dehydrate, stain, and counterstain.
4. Rinse in 95 per cent alcohol.

5. Transfer to Hygrobutol by the addition and subtraction method.
6. Make 2 changes of pure Hygrobutol.
7. Infiltrate with balsam highly diluted with Hygrobutol.
8. Concentrate by slow evaporation.
9. Mount on a slide as soon as thick enough, and apply a cover glass.
10. Dry.

Maceration of Wood

Schultze's Method

1. Boil fragments in 50 per cent nitric acid to which is added crystals of potassium chlorate until disintegration commences.
2. Wash by agitation and decanting.
3. Dehydrate in 95 per cent and 100 per cent alcohol. Work rapidly, as one is not concerned with plasmolysis.
4. Transfer to xylol.
5. Stain by the addition of a clove oil solution of the dye.
6. Remove excess stain with xylol to which a trace of 100 per cent alcohol has been added.
7. Wash in pure xylol.
8. Mount in balsam.

Jeffrey's Method

1. Boil fragments of wood in water to eliminate air.
2. Macerate for from 24 to 48 hours at 35° C. in 1:1 mixture of 10 per cent nitric and 10 per cent chromic acid.
3. Wash, stain, and mount as described in Steps 2 to 8 for Schultze's method.

Smear Method for Pollen Grains

Microsporocytes and even pollen grains in the tetrad stage will adhere to a clean glass slide without the use of any additional adhesive. Cut anthers crosswise or split lengthwise, using only a small amount of material on one slide.

Belling's Iron-Acetocarmine Temporary Method

1. Cut anthers on a chemically clean slide.
2. Dry with blotting paper.
3. Add a drop of iron-acetocarmine for 2 minutes.

4. Remove with blotting paper.
5. Add a fresh drop of iron-acetocarmine.
6. Press out the microsporocytes.
7. Tease out the anther walls.
8. Put on cover glass.
9. Seal with petroleum jelly or paraffin on a hot rod.
10. Leave for several days or weeks for completion of staining.
11. Press out further during examination if desired. The results are excellent but temporary. A blue or green screen (as from copper sulfate solution) in front of the microscope light improves the image.

Formula for Iron-Acetocarmine

Acetic acid, glacial	-----	90 c.c.
Distilled water	-----	110 c.c.
Carmine	-----	1 Gm.

Add the carmine to the acetic acid at the boiling point.

Cool on ice.

Add 1 or 2 drops of ferric salt (such as acetate) to produce a dark wine-red color.

Belling's Permanent Method

1. Put portion of cut anther on chemically clean slide.
2. Squeeze out the microsporocytes with one sweep of another clean slide or a flat-ground, straight-edged scalpel, increasing pressure during the stroke.
3. Invert immediately and horizontally into Navashin's fixing solution in a large Petri dish, supporting the ends of the slide on glass rods or blank slides.
4. Fix for 3 to 12 hours.
5. Rinse for 10 minutes in Navashin's solution A.
6. Remove anther fragments.
7. Dehydrate through 15, 30, 50, and 70 per cent alcohols.
8. Leave overnight in 70 per cent alcohol.
9. Mordant in ferric ammonia alum, 1 per cent in 70 per cent alcohol, for 24 hours.
10. Rinse in 70 per cent alcohol.
11. Soak in 70 per cent alcohol for from 15 minutes to 3 hours.
12. Stain in brazilin or hematoxylin, 0.5 per cent in 70 per cent alcohol for from 2 to 24 hours.

13. Rinse in 70 per cent alcohol.
14. Differentiate in ferric ammonia alum, 1 per cent in 70 per cent alcohol, for from 1 minute to 3 hours.
15. Rinse in 70 per cent alcohol.
16. Transfer to 90 per cent alcohol.
17. Absolute alcohol.
18. Absolute alcohol-thin cedar oil, 1:1.
19. Xylol-thin cedar oil, 1:1.
20. Xylol.
21. Mount in immersion cedar oil.

Taylor and Kaufmann's Method

Taylor and Kaufmann's method differs from Belling's in the fixing solution, in the use of iron alum hematoxylin prior to partial dehydration, in that it requires a shorter staining period, and in the use of balsam as a mounting medium instead of cedar oil.

1. Put portion of cut anther on chemically clean slide.
2. Smear.
3. Invert for 15 minutes into the following fixing solution:

Acetic acid, 10 per cent	2 c.c.
Chromic acid, 10 per cent	0.2 c.c.
Osmic acid, 2 per cent, in 2 per cent chromic acid	1.5 c.c.
Distilled water	8.3 c.c.
Add 1 per cent of maltose.	

4. Wash in changes of water for 1 hour.
5. Remove anther fragments.
6. Bleach in hydrogen peroxide.
7. Rinse in water.
8. Mordant 45 minutes in iron ammonia alum.
9. Wash 15 minutes in running water.
10. Stain in 0.5 per cent hematoxylin solution for from 20 to 30 minutes.
11. Differentiate in iron ammonia alum.
12. Wash 1 hour.
13. Dehydrate in alcohol series.
14. Clear through xylol-absolute alcohol in 4 stages.
15. Transfer to pure xylol.
16. Concentrate very dilute xylol balsam on the slide by evaporation before putting the cover glass in place.

Staining Pollen Tubes in Stigma Tissue Buchholz and Blakeslee's Method

1. Soften the stigmas by scalding.
2. Dissect if large.
3. Stain in aqueous acid fuchsin.
4. Mount in lactic-glycerin solution.

Lactic acid	-----	20 c.c.
Glycerin	-----	55 c.c.
Water	-----	25 c.c.

5. Smear by pressure on the cover glass.
6. For permanent mounts, transfer to glycerin jelly to which 20 per cent lactic acid has been added.

Freehand Sections

Hold the material between the thumb and first finger of the left hand. Add water to wet the razor blade, which should be plane convex. Use the length of the first finger as a guide for the razor to obtain parallel sections or cut between the thumb and first finger to obtain median bisection of small objects, such as moss capsules and archegonial receptacles of liverworts.

Sectioning in Pith

For the temporary embedding of nonrigid tissues, a pith stick can be used. The stick, soaked in water or glycerin-alcohol mixture, is split lengthwise, the soft tissue placed within the split, and the entire mass sectioned in the sliding microtome. The small fragments of pith can readily be eliminated when mounting the material.

An inexpensive hand microtome can be improvised by screwing a pith stick through a hexagonal nut, the top of which is polished as a guide for the razor.

Sectioning and Staining Woody Stems

1. Section in a sliding microtome without prior embedding procedure.
- Wet blade with 1:1 glycerin-alcohol mixture.
2. Fix the sections in a Syracuse watch glass full of formalin-acetic alcohol.
3. Wash in water for aqueous stains or 50 per cent alcohol for alcoholic stains.

Rather than transferring the individual sections through a series of watch glasses, since but small amounts of material are necessary, it is simpler and easier to use one watch glass, discarding the previous portion. Use a medicine dropper to change the solutions, thereby stirring the solution and making gradual transitions from one solution to another and preventing plasmolysis.

4. Stain with a basic dye.
5. Wash.
6. Counterstain with an acid dye.
7. Wash.
8. Increase the alcoholic concentration to 95 per cent.
9. Transfer the sections to 100 per cent alcohol in a covered watch glass.
10. Add xylol and increase the concentration to 100 per cent xylol.
11. Mount the sections directly in Canada balsam or gum dammar on a slide and apply a cover glass, using care to exclude any air bubbles.
12. Dry on a hot plate or in an oven at about 50° C.

Alternative Procedure:

1. Section.
2. Fix.
3. Wash.
4. Stain with an alcoholic solution of a basic dye.
5. Wash in 50 per cent alcohol.
6. Increase the concentration of alcohol to 100 per cent.
7. Add xylol and increase the concentration to 100 per cent.
8. Counterstain with an acid stain in clove oil.
9. Clear in pure clove oil for from 15 to 20 minutes.
10. Wash in xylol containing a trace of 100 per cent alcohol.
11. Wash in pure xylol.
12. Mount in balsam or gum dammar.
13. Dry.

Frozen Section Method

In botanical technic, the frozen section procedure is applicable only to soft or gelatinous materials, such as red and brown algae. See page 76 for details of the procedure. Haupt's fixative can be used as well as celloidin for attaching the sections to slides, or they may be handled in a Syracuse watch glass.

Celloidin Section Method

The celloidin section procedure is used in botanical technic only where embedding in paraffin is not effective because the material is either too delicate or too hard. Superior sections of hard stems and roots can be obtained by this method.

Embedding is even slower than for zoological materials. The schedule by Jeffrey is given below. For sectioning, see page 117.

Jeffrey's Embedding Procedure

1. Fix.
2. Desilicify if necessary.
3. Wash.
4. Dehydrate through an ethyl or butyl alcohol series.
5. Transfer from 100 per cent alcohol to ether-alcohol mixture for 24 hours.
6. Transfer to 2 per cent celloidin in a nearly full shell vial and stopper. Clamp or wire the stopper and place in a paraffin bath at 50° to 60° C. for from 12 to 18 hours.
7. Chill in cold water and return the solution to stock bottle.
8. Repeat with 4 per cent celloidin.
9. Repeat with 6 per cent celloidin.
10. Repeat with 8 per cent celloidin.
11. Repeat with 10 per cent celloidin.
12. Repeat with 12 per cent celloidin.
13. Repeat with 14 per cent celloidin.
14. Repeat with 16 per cent celloidin.
15. Repeat with 18 per cent celloidin.
16. Transfer to 20 per cent celloidin and increase concentration by adding chips of dry celloidin until a firm texture is attained.
17. Harden the block in chloroform for 12 hours.
18. Transfer to glycerin-alcohol (95 per cent) mixture for several days prior to cutting.

The simpler infiltration procedure given on page 117 would be better for one's first attempt at the celloidin method.

Paraffin Section Method

In botanical, as in histological technic, xylol has been displaced as a medium of approach to paraffin. Dehydration in butyl alcohol eliminates a second fluid entirely and at the same time keeps tissues softer.

Zirkle's Normal Butyl Alcohol Method

1. Fix and evacuate.

2. Wash.

Dehydrate in the following series, using enough liquid to just cover the material in a shell vial:

		<i>Water</i>	<i>Ethyl Alcohol</i>	<i>Normal Butyl Alcohol</i>	<i>Time</i>
3.	5 per cent	95	5	0	2 hours
4.	11 per cent	89	11	0	2 hours
5.	18 per cent	82	18	0	2 hours
6.	30 per cent	70	30	0	2 hours

Enter the series at this point from 50 per cent alcoholic fixatives and 50 per cent alcohol wash.

7.	50 per cent	50	40	10	2 hours overnight
8.	70 per cent	30	50	20	3 hours
9.	85 per cent	15	50	35	3 hours
10.	95 per cent	0	45	55	3 hours
11.	100 per cent tinged red with erythrosin.				3 changes of 3 hours each

(absolute) 25 75

- | | | | |
|-----|--------------------|---|-----|
| 12. | Pure butyl alcohol | 0 | 100 |
|-----|--------------------|---|-----|
13. Transfer the material to a vial two-thirds full of paraffin solidified in place, and cover with normal butyl alcohol.
14. Place in paraffin oven until the paraffin melts.
15. Decant and make 3 changes of paraffin, using a paraffin-rubber mixture for the last change.
16. Embed in the paraffin-rubber mixture.

Many good mixtures are available under various trade names. One formula by Hance is given below.

Hance's Paraffin-Embedding Mixture

Paraffin-rubber stock:

Paraffin -----	100 c.c.
Rubber, crude -----	20 Gm.

Heat to the smoking point for 3 to 4 hours.

Paraffin-embedding mixture:

Paraffin, melting point 53° C. -----	100 Gm.
Paraffin-rubber stock -----	3 Gm.
Beeswax -----	1 Gm.

Filter through paper while hot. In paraffin oven at 56° C.

Johansen's Tertiary Butyl Alcohol Method

The procedure for Johansen's tertiary butyl alcohol method is identical with Zirkle's, excepting that tertiary butyl alcohol is used in place of normal butyl alcohol.

Keep above 17° C. to prevent solidification.

After dehydration, the material is transferred to a 1:1 mixture of tertiary butyl alcohol and paraffin oil for an hour before continuing with pure paraffin.

Even better results are claimed as compared with those obtained from normal butyl alcohol.

Embedding in Paraffin:

Aluminum foil (0.006 inch) folded over a block of wood forms an excellent substitute for paper in making embedding boxes.

Use a Huettner oven having an overhead heating element, and an additional feature which might be adapted to other ovens, namely, a coarse mesh wire screen supported over a shallow work shelf. Instead of transferring the embedding box to cold water, and thereby taking a chance of disarranging the pieces of material, a glass of ice water is dumped into the tray, causing instant congealing of the bottom layer, whereupon it can be moved without danger, to a dish of ice water, in which after a moment it can be completely submerged.

Sectioning in Paraffin:

Follow the instructions on page 87.

Additional Suggestions:

1. Soak blocks containing plant stems and similar woody materials in water for several days prior to sectioning.
2. Use a straight-edged scalpel for trimming blocks.
3. Where the holder has a good coating of paraffin, an old knife or scalpel heated in a burner and passed between the block and the holder will seal the two together.

4. After straightening the ribbon, by laying the latter on either permanent slides or larger glass plates flooded with water, a preliminary microscopic examination can be made in order to pick out median sections of plant structures, eliminating the staining of worthless blank sections.

5. An ink mark or number in the upper right or "thumb" corner of the slide is convenient in telling the top of the slide in subsequent staining operations. Two formulas are given below:

Hubbert's Ink for Writing on Glass.

Shellac, 13 per cent alcoholic	3 parts
Borax, 13 per cent aqueous	5 parts

Mix drop by drop.

Heat to clear if necessary.

Add methylene blue to color the mass a deep blue.

Otis' Ink for Writing on Glass.

Glue, 15 per cent	100 c.c.
Potassium dichromate	in excess

Expose to light for a week or longer.

Filter.

Rub stick India ink into small portions of stock solution.

Keep in a sealed balsam bottle.

Haupt's Adhesive

Haupt's fixative is superior in its adhesive properties for stem sections and similar material.

Gelatin, pure (Knox)	1 Gm.
Distilled water	100 c.c.
Phenol crystals	2 Gm.
Glycerin	15 c.c.

Dissolve the gelatin in water at 30° C. Add phenol and glycerin. Stir and filter.

Use sparingly.

Spread evenly with a clean finger.

Remove any excess.

Float sections on slide in 2 per cent formaldehyde over a warming plate.

Pour off the excess solution as soon as the sections have straightened out.

Dry in the air, not on the plate.

Place the dry slides in a slide box under the hood of the slide hot plate or an oven, with a dish of concentrated (40 per cent) formaldehyde. The fumes will turn the gelatin back into the form from which it came and firmly attach the sections to the slide.

Staining Sections on Slides

Processing of sections attached to slides is attained through a series of dishes, preferably square Coplin jars, rather than by the addition-subtraction method. Only in commercial work is there any advantage in slide baskets and large dishes for staining. If slides are to be left for any length of time in one solution, they are placed in the grooves of the Coplin jar, otherwise they are gently agitated at right angles to the grooves for about 15 seconds, slowly withdrawn to permit simultaneous drainage, and transferred to the next in series, touching the edge of the slide to paper toweling before entering absolute alcohol. For convenience, the jars are arranged in a semicircle in front of the operator. For best results, replace solutions before they are worn out; that is, after 50 or 60 slides, or 25 through absolute alcohol.

Although designed for another purpose, a Diffenbach clamp is superior to any forceps for holding a slide while passing it through the series.

Tuan's Picric Acid Method for Differentiation

Make up a stock solution of 5 per cent picric acid in 95 per cent alcohol. Six cubic centimeters of stock solution added to sufficient alcohol for each Coplin jar gives a total of 60 c.c., with a picric acid concentration of 0.5 per cent.

Picric Acid-Alcohol Series for Differentiation

	<i>Stock Solution</i>	<i>95 Per Cent Alcohol</i>	<i>Water</i>	<i>Total</i>
15 per cent	6 c.c.	4 c.c.	50 c.c.	60 c.c.
35 per cent	6 c.c.	16 c.c.	38 c.c.	60 c.c.
50 per cent	6 c.c.	26 c.c.	28 c.c.	60 c.c.
70 per cent	6 c.c.	38 c.c.	16 c.c.	60 c.c.
85 per cent	6 c.c.	48 c.c.	6 c.c.	60 c.c.
95 per cent	6 c.c.	54 c.c.	0	60 c.c.

Staining Schedule: Safranine vs. Fast Green

(Including Tuan's Picric Acid Series for Differentiation)

1. Xylol for several minutes to dissolve paraffin out of sections. Proceed through the series:
2. Second xylol.
3. Xylol-absolute alcohol (1:1).
4. 100 per cent alcohol.
5. 95 per cent alcohol.
6. 85 per cent alcohol.
7. 70 per cent alcohol.
8. 50 per cent alcohol.
9. Stain overnight or longer in safranine, 1 per cent in a mixture of equal parts of 95 per cent alcohol and a saturated solution of aniline oil in water.
10. Rinse in a larger dish of 50 per cent alcohol. Proceed through the series:
11. 50 per cent picric acid-alcohol mixture.
12. 70 per cent picric acid-alcohol mixture.
13. 85 per cent picric acid-alcohol mixture.
14. 95 per cent picric acid-alcohol mixture.
15. 95 per cent alcohol, with a few drops of ammonium hydroxide.
16. Touch edge of slide to paper toweling. Inspect with microscope.
17. 100 per cent alcohol.
18. Dry the bottom of the slide.
19. Hold the slide level and apply counterstain of fast green in clove oil from a dropper bottle. Stain 5 to 10 seconds.
20. Return the dye solution to the dropper bottle.
21. Rinse with a dropper over a dish of waste clove oil diluted with xylol and absolute alcohol from the contents of the dish.
22. Rinse with a fresh supply of waste clove oil diluted with xylol and absolute alcohol out of a dropper bottle.
23. Touch edge of slide to paper toweling. Inspect with microscope.
24. Transfer slide to pure clove oil.
25. Xylol with a trace of 100 per cent alcohol.
26. Pure xylol for 5 minutes.
27. Mount in dilute balsam and apply cover slip.
28. Dry on a hot plate or in an oven at about 50° C.

Staining Schedule: Heidenhain's Hematoxylin vs. Orange G

1. Xylol for several minutes to dissolve paraffin out of sections. Proceed through the series:
2. Second xylol.
3. Xylol-100 per cent alcohol (1:1).
4. 100 per cent alcohol.
5. 95 per cent alcohol.
6. 85 per cent alcohol.
7. 70 per cent alcohol.
8. 50 per cent alcohol.
9. 35 per cent alcohol.
10. 12 per cent alcohol.
11. Wash in running water.
12. Rinse in distilled water.
13. Mordant for from 45 minutes to 3 hours in 2 per cent ferric ammonium sulfate acidified with 1 per cent glacial acetic acid and 0.1 per cent sulfuric acid.
14. Wash in running water for from 1 to 3 minutes.
15. Stain for 1 to 2 hours or longer in ripened, freshly filtered 0.5 per cent aqueous hematoxylin to which is added a few drops of saturated solution of lithium carbonate.
16. Wash in running water.
17. Destain in 2 per cent acidified ferric ammonium sulfate.
18. Complete destaining under the microscope with a fresh drop of solution.
19. Short stop the destaining in running water.
20. Check on the microscope for further destaining if necessary.
21. Wash 40 minutes.
22. Dip in ammonia water for blue instead of black tones if the former are preferred.
23. Dehydrate through an alcohol series.
24. Counterstain with 95 per cent alcoholic solution of orange G.
25. Rinse in 95 per cent alcohol.
26. Complete dehydration in 100 per cent alcohol.
27. Transfer through 100 per cent alcohol-xylol mixture.
28. Pure xylol for 5 minutes.
29. Mount in dilute balsam and apply cover slip.
30. Dry on hot plate or in oven at about 50° C.

Flemming's Triple Stain for Use in Cytology
Safranine, Gentian Violet, and Orange G

1. Stain with safranine, as given on page 293.
2. Rinse in 50 per cent alcohol for 1 minute.
3. Rinse in water for 1 minute.
4. Supplementary stain in 1 per cent aqueous gentian violet for from 3 to 30 minutes.
5. Rinse in water for a few seconds.
6. Dehydrate in 95 per cent alcohol for 5 seconds.
7. Dehydrate in 100 per cent alcohol for 10 seconds.
8. Counterstain with orange G in clove oil, as described on page 294 for fast green in clove oil.
9. Transfer to xylol.
10. Mount in balsam and apply cover slip.

The objection to both this procedure and Conant's modification of it is that dehydration cannot be by gradual stages, due to the solubility of the gentian violet.

Conant's "Triarch" Quadruple Stain
Safranine, Gentian or Crystal Violet, Fast Green and
Methyl Orange (Gold Orange)

1. Stain in safranine for from 2 to 24 hours, as given on page 293.
2. Rinse thoroughly in 2 changes of water.
3. Stain in gentian violet, saturated aqueous solution, for about 1 minute.
4. Rinse in 1 change of water.
5. Dehydrate through 2 changes of absolute alcohol, about 1 minute in each.
6. Agitate slides quickly in fast green, 1 per cent in absolute alcohol.
7. Apply methyl orange, saturated solution in clove oil, as described on page 294, for fast green in clove oil.
8. Transfer to xylol.
9. Mount in balsam and apply cover glass.

In commercial production, Conant uses a series of methyl orange-clove oil baths in the final stain, eliminating the vessel of fast green as soon as sufficient is carried over into the first methyl orange-clove oil bath. It is, therefore, possible to combine Steps 6 and 7 by

using one application of a mixture of methyl orange in clove oil and fast green in clove oil in the proportions of about 3 to 1.

With this stain lignified structures are colored red; starch grains and nuclei, violet; cellulose structures, green; background, orange.

Staining Fungus Mycelium in Wood Sections Mueller's Application of Pianese's Stain

1. Run sections down to 35 per cent alcohol.
2. Stain in Pianese IIIb stain for from 15 to 45 minutes.

Martius yellow -----	0.01 Gm.
Malachite green -----	0.50 Gm.
Acid fuchsin -----	0.10 Gm.
Distilled water -----	150 c.c.
Alcohol, 95 per cent -----	50 c.c.

3. Rinse in water.
 4. Differentiate in acidified alcohol.
 5. Dehydrate, clear, and mount in balsam.
- Host tissue stains green and mycelium a deep pink.

Cartwright's Method

1. Stain with 1 per cent aqueous safranine.
2. Wash in water.
3. Counterstain with:

Aniline blue, saturated aqueous solution -----	25 parts
Picric acid, saturated aqueous solution -----	100 parts

4. Heat over a small flame until the dye begins to simmer.
 5. Wash.
 6. Dehydrate, clear in clove oil, and mount in balsam.
- Host tissue stains red and mycelium blue.

Steil's Antherozoid Stain

1. Place over osmic acid fumes for 2 minutes to kill.
2. Air dry.
3. Bleach in hydrogen peroxide for several minutes.
4. Soak in a saturated solution of tannic acid for from 10 minutes to several hours.
5. Stain in safranine and counterstain in fast green, as described on page 293.

PART X

HISTOPATHOLOGICAL METHODS OF THE CENTRAL NERVOUS SYSTEM

The fixing and staining methods in neuropathological laboratories are exceedingly numerous, and in many instances complicated and burdensome.

The following methods have been found of practical use.

NUCLEAR STAINS

Hematoxylin Eosin Stain

See method on page 141.

Ethyl Violet-Orange G Stain

1. Fix tissues in Zenker's fluid and make paraffin sections in usual manner. Formol-fixed paraffin sections can also be used if mordanted in Zenker's fluid for 3 days.
2. Fix sections in 3 per cent potassium bichromate solution.
3. Treat in Lugol's solution for 5 minutes.
4. Wash in water.
5. Stain in neutral ethyl violet-orange G for from 12 to 24 hours.
6. Blot sections and wash in anhydrous acetone.
7. Dip in toluol and flood slide with pure clove oil.
8. Differentiate in a mixture of 3 parts of clove oil and 1 part of 95 per cent alcohol.
9. Rinse in clove oil and wash in toluol.
10. Blot and clear in 2 changes of pure xylol.
11. Mount in gum dammar.

Formula of Ethyl Violet-Orange G (Stock Solution)

Add 1 Gm. ethyl violet and 0.5 Gm. orange G to 100 c.c. distilled water and stir to insure thorough mixing. Place in or on an oven to precipitate, which requires from 12 to 24 hours. Decant the supernatant fluid and wash the precipitate several times with distilled water. Place in or on an oven again to dry. Dissolve in absolute alcohol.

Use 1 part of stock solution to 3 parts of 20 per cent alcohol.

Schultze-Stohr Nerve Cell Stains for Ganglion Cells

1. Fix tissues in 10 per cent formaldehyde for 48 hours or longer.
2. Cut frozen sections 30 microns thick and place them in distilled water.
3. Place in solution of 0.5 part of normal sodium hydroxide in 50 parts of distilled water for 24 hours to remove granular precipitate. (For normal sodium hydroxide, dissolve 4 Gm. of chemical in 100 c.c. distilled water.)
4. Wash in large quantity of distilled water for 1 hour, changing the water several times.
5. Place in 0.5 per cent aqueous silver nitrate solution for 16 hours or longer. The solution must remain clear when sections are placed in it.
6. Reduce in hydroquinone solution made up as follows:

Hydroquinone -----	2.5 Gm.
Distilled water -----	100 c.c.
Formaldehyde, commercial -----	5 c.c.

The stock solution keeps for 3 months in a dark place. For reducing sections, add 20 parts of distilled water to 1 part of the stock solution. Watch the reduction process under the microscope.

7. Wash quickly in distilled water and blot.
 8. Dehydrate in several changes of absolute alcohol.
 9. Clear in carbolxylol for 5 minutes.
 10. Wash in 2 changes of pure xylol.
 11. Mount in gum dammar.
- Cell bodies appear brown or black; dendrites, deep black.

For Brain Tissue

1. Cut frozen sections 30 microns thick and place in distilled water.
2. Place in sodium hydroxide solution for 24 hours (6 parts normal sodium hydroxide to 50 parts distilled water).
3. Wash in distilled water for 1 hour, changing water frequently.
4. Place in 2 per cent silver nitrate solution 16 hours or longer. There must be no clouding of solution.
5. Reduce in hydroquinone solution for a few seconds, and examine under the low power of microscope. When solution becomes cloudy, use fresh solution.
6. Wash in 2 changes of distilled water.
7. Dehydrate in 3 changes of absolute alcohol.

8. Clear in carbolxylol, then in 2 changes of xylol.
9. Mount in gum dammar.

For Basal Ganglia

1. Cut frozen sections 30 microns thick and place them in sodium hydroxide solution (10 parts normal sodium hydroxide to 50 parts distilled water).
2. Wash in distilled water for 1 hour, changing the water frequently.
3. Place in a 10 per cent aqueous silver nitrate solution for from 16 to 24 hours.
4. Reduce in hydroquinone solution for a few seconds. Examine under microscope.
5. Wash in distilled water.
6. Dehydrate in 3 changes of absolute alcohol.
7. Clear in carbolxylol, then in 2 changes of pure xylol.
8. Mount in gum dammar.

For Cerebellum

1. Cut frozen sections 30 microns thick and place them in sodium hydroxide solution for 24 hours (2 parts normal sodium hydroxide to 50 parts distilled water).
2. Wash in distilled water for 1 hour, changing the water frequently.
3. Place sections in 0.25 per cent aqueous solution of silver nitrate for from 16 to 24 hours.
4. Reduce in hydroquinone solution for a few seconds, examining sections under microscope.
5. Wash in distilled water.
6. Dehydrate in 3 changes of absolute alcohol.
7. Clear in carbolxylol, then in 2 changes of pure xylol.
8. Mount in gum dammar.

For Medulla, Spinal Cord, and Sympathetic Ganglia

1. Cut frozen sections 30 microns thick and place in sodium hydroxide solution for 24 hours (10 parts normal sodium hydroxide to 50 parts distilled water).
2. Wash in distilled water for 4 hours, changing the water frequently.
3. Place sections in 10 per cent aqueous solution of silver nitrate for from 16 to 24 hours.

4. Reduce in hydroquinone solution for a few seconds, examining sections under microscope.
5. Wash in distilled water.
6. Dehydrate in 3 changes of absolute alcohol.
7. Clear in carbolxylol, then in 2 changes of pure xylol.
8. Mount in gum dammar.

For Glia

1. Cut frozen sections 30 microns thick and place them in sodium hydroxide for 24 hours (0.5 part normal sodium hydroxide to 50 parts distilled water).
2. Wash in distilled water for 48 hours, changing the water frequently.
3. Place sections in 0.5 per cent aqueous solution of silver nitrate for from 16 to 24 hours.
4. Reduce in hydroquinone solution for a few seconds, examining sections under microscope.
5. Wash in distilled water.
6. Dehydrate in 3 changes of absolute alcohol.
7. Clear in carbolxylol, then in 2 changes of pure xylol.
8. Mount in gum dammar.

For Peripheral Nerves

1. Cut frozen sections 30 microns thick and place in a sodium hydroxide solution for 24 hours (10 parts normal sodium hydroxide in 50 parts distilled water).
2. Wash in distilled water for 1 to 2 hours, changing frequently.
3. Place in 10 per cent aqueous solution of silver nitrate for 24 hours.
4. The reduction is very rapid and difficult to control. Therefore, dilute the stock solution of hydroquinone 100 times with distilled water, and watch the process under the microscope.
5. Wash in distilled water.
6. Dehydrate in 3 changes of absolute alcohol.
7. Clear in carbolxylol, then in 2 changes of pure xylol.
8. Mount in gum dammar.

When the nerve begins to appear, during Step 4, the section should at once be put into distilled water. If kept too long in the reduction fluid, the rest of the section stains the same dark color. If kept too short a time, the nerves cannot be identified; they stain incompletely or not at all. After the sections have been taken out

of the reducing fluid and placed in distilled water, they cannot be returned to any fluid previously used. Thus, sections should be taken out frequently from the reducing fluid and examined under the microscope to find the nerves.

NISSL GRANULE STAINS

Nissl's original method is long and complicated. The following methods may be employed advantageously to demonstrate Nissl granules:

Toluidine Blue Method

1. Fix very thin blocks of tissue in 95 per cent alcohol for 2 to 4 days.
2. Make thin paraffin or celloidin sections in the usual manner.
3. Stain in 1 per cent aqueous solution of toluidine blue overnight at room temperature, or 2 hours in oven at 55° C.
4. Rinse rapidly in water.
5. Dehydrate for 1 minute in 95 per cent alcohol.
6. Differentiate in the following solution, controlling the extraction of stain under a microscope:

Creosote, pure	5 c.c.
Cajuput oil	4 c.c.
Xylol	5 c.c.
Alcohol, absolute	15 c.c.

7. Rinse in absolute alcohol.
8. Clear in 2 changes of xylol.
9. Mount in gum dammar.

Nissl granules stain blue, against an unstained cytoplasmic background; nuclei remain nearly colorless.

Cyanin Stain

1. Cut frozen sections 5 to 10 microns thick.
2. Transfer to slide.
3. Dehydrate and fix with celloidin.
4. Place in stain for from 24 to 36 hours.

Chrome alum	10 Gm.
Distilled water	200 c.c.

Mix and heat with 0.3 Gm. cyanin.

Boil for 20 minutes. Make up to original volume with distilled water. This keeps indefinitely. It should be filtered just before use.

5. Rinse in water.
6. Differentiate in 95 per cent alcohol. Change 4 or 5 times.
7. Dehydrate rapidly in absolute alcohol.
8. Clear in xylol.
9. Mount in gum dammar.

Cresyl (Echt) Violet Stain

1. Make thin frozen or paraffin sections in the usual manner.
2. Stain in 1 per cent aqueous solution of cresyl (echt) violet (filtered) for 36 hours at room temperature.
3. Wash in distilled water.
4. Differentiate in 95 per cent alcohol until the background is white or nearly so.
5. Complete dehydration in 3 changes of absolute alcohol.
6. Clear in 4 changes of pure xylol.
7. Mount in gum dammar.

NERVE FIBER STAINS

Bielschowsky Silver Stain

1. Fix tissues in 15 per cent formaldehyde for 48 hours or longer.
2. Wash for several hours under running water.
3. Cut frozen sections about 10 to 15 microns thick.
4. Wash thoroughly in distilled water.
5. Place in pyridine for from 24 to 48 hours.
6. Wash in many changes of distilled water until pyridine is completely eliminated. Transfer into 3 per cent silver nitrate for 24 hours in a dark place at room temperature.
7. Take the sections one by one from the silver nitrate and wash quickly in distilled water. Transfer into ammoniacal silver for about 10 minutes until they become yellowish brown. Ammoniacal silver solution is made as follows:

Silver nitrate, 10 per cent	-----	30 c.c.
Sodium hydroxide, 40 per cent	-----	40 drops

A precipitate forms. Wash the precipitate a dozen times with distilled water (use about a liter of water). Add 50 c.c. distilled water to the washed precipitate, and then ammonium hydroxide drop by drop until it is dissolved, but avoid excess. Make up to 150 c.c. with distilled water. Keep in a dark place.

8. Wash quickly in distilled water.

9. Reduce in 20 per cent formaldehyde in distilled water. If working with a number of sections, it is advisable to retransfer them into a fresh bath of 20 per cent formaldehyde. Reduction takes place at the end of half an hour or less.

10. Wash in distilled water. Tone in 2 per cent gold chloride. Acidify the gold bath slightly with acetic acid to obtain a faintly purple background, or neutralize with a few drops of a diluted solution of sodium or lithium carbonate for a grayish background. A slightly acid solution of chloroplatinic acid may be used instead of gold chloride.

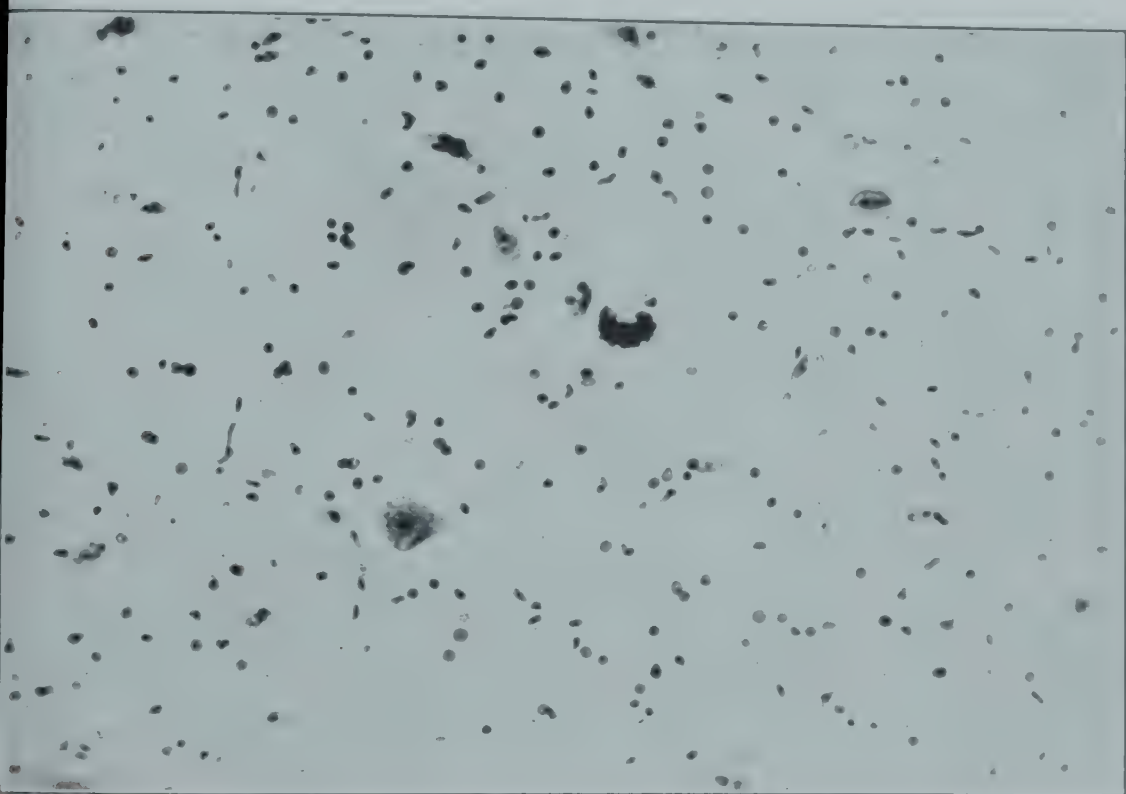


Fig. 117.—Nerve cell stain. Cyanin method. Frozen section.

11. Wash in distilled water.

12. Treat in 5 per cent sodium hyposulfite solution.

13. Wash in tap water.

14. Dehydrate completely in 3 changes of absolute alcohol.

15. Clear in carbolxylol for 5 minutes. Wash in 2 changes of pure xylol. Mount in gum dammar.

Neurofibrils stain grayish back.

An excellent method to demonstrate nerve fibers.

Cajal's Nerve Fiber Stain

Impregnation method for unmyelinated fibers in formaldehyde-fixed tissues, Cajal's nerve fiber stain is absolutely constant and especially applicable to cerebellum.

1. Pieces of nerve tissue, especially cerebellum, are fixed in 15 per cent formaldehyde for 2 weeks.
2. Cut frozen sections 15 to 20 microns thick.
3. Place in 10 per cent formaldehyde. Wash rapidly in 2 changes of distilled water.

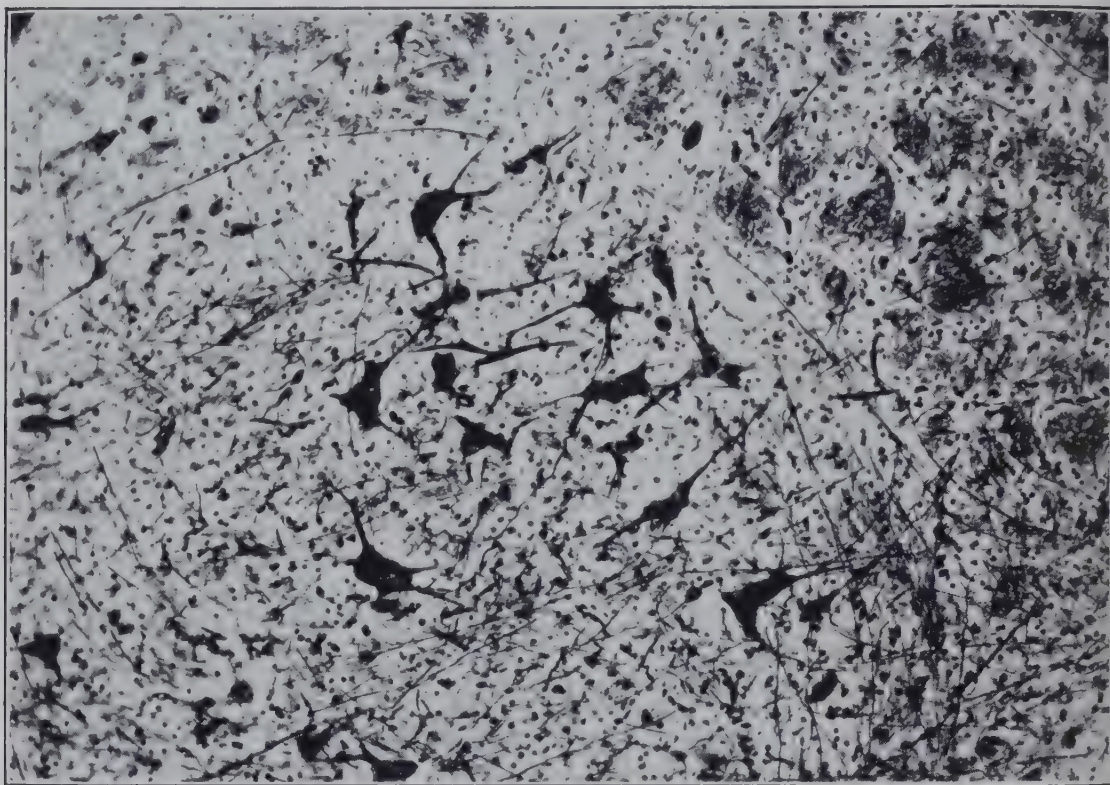


Fig. 118.—Anterior horn cells of spinal cord. Reduced silver method (Cajal).
Frozen section.

4. Place immediately into :

Silver nitrate solution, 2 per cent	-----	10 c.c.
Pyridine, pure	-----	5 drops

Leave in this silver solution 3 to 4 hours if the tissues are fresh. Sections from old material should be left from 20 to 40 hours or until they are a tobacco color. Keep them in a dark place. If in a hurry, heat the solution for a few minutes.

5. Place in 10 c.c. of 95 per cent alcohol for 1 minute. If the impregnation is not intense (dark brown), add 2 or 3 drops of 2 per cent silver nitrate solution to the alcohol bath.

6. Place sections without washing into the following reducer:

Hydroquinone, pure -----	0.2 Gm.
Formaldehyde (Merck) -----	30 c.c.
Distilled water -----	70 c.c.

Leave in this solution for about 20 minutes.

7. Wash in distilled water.

8. Dehydrate in 3 changes of absolute alcohol.

9. Clear in carbolxylol for 5 minutes. Wash in 2 changes of pure xylol. Mount in gum dammar. Unmyelinated fibers and chromatin stain black. If in Step 5 the sections are left in 95 per cent alcohol for 6 hours or longer, the medullated fibers are well impregnated, but the cells remain unstained.

In Step 5, only one section at a time should be placed in the alcohol bath.

NERVE ENDINGS AND PLEXUSES

Gold Chloride Method

Impregnation with gold chloride, when successful, produces a very good picture of nerve endings and nerve plexuses.

1. Cut thin slices of fresh tissue.

2. Wash and place in physiological salt solution, then in filtered lemon juice until they become clear. This requires from 5 to 10 minutes.

3. Rinse with distilled water and place in 1 per cent solution of gold chloride (about 10 times their volume) in the dark for from 30 to 40 minutes.

4. Rinse with distilled water and place in a mixture of 1 part of formic acid in 3 parts of distilled water. Keep in dark for from 24 to 48 hours, during which time the gold is reduced.

5. Wash in many changes of distilled water.

6. Tease apart the tissue block and mount in glycerin jelly, or embed in paraffin in the usual manner and section and mount in gum dammar.

Nerve endings and plexuses stain violet to black.

MITOCHONDRIA STAIN**Bailey-Davis Method**

1. Fix tissues in Regaud's fluid or 10 per cent formaldehyde and mordant a week or 10 days in 3 per cent potassium bichromate solution.
2. Wash, dehydrate, and embed in paraffin in usual manner.
3. Cut sections 3 to 5 microns thick.
4. Place in 0.25 per cent potassium permanganate solution for 30 minutes or longer (up to 2 hours) or in 1 per cent solution for from 20 to 30 minutes.
5. Wash in distilled water.
6. Bleach in 1 per cent oxalic acid solution until colorless.
7. Wash in distilled water very thoroughly.
8. Stain in Mallory's phosphotungstic acid hematoxylin for from 12 to 24 hours.
9. Wash out excess stain in 95 per cent alcohol.
10. Dehydrate in 2 changes of absolute alcohol.
11. Blot and clear in 2 changes of pure xylol.
12. Mount in gum dammar.

See Mallory's phosphotungstic acid hematoxylin formula, page 255.

Cowdry's Method

1. Fix tissues in Regaud's mixture (3 per cent potassium bichromate, 20 c.c., and formaldehyde 5 c.c.). The commercial formaldehyde may profitably be neutralized by saturation with magnesium carbonate. The mixture may be applied by immersion or injection, the latter being recommended for large objects. It should be changed every day for 4 days.

Mordant for 8 days in 3 per cent potassium bichromate, changing every second day.

2. Wash in running water overnight.
3. Dehydrate, clear, and embed in usual manner.
4. Cut paraffin sections and pass slides to water in usual manner.
5. Place in 1 per cent potassium permanganate solution for 30 seconds or longer.
6. Place in 5 per cent oxalic acid solution for 30 seconds.
7. Rinse in several changes of distilled water for about 1 minute. Incomplete washing prevents staining with fuchsin.

8. Stain in Altman's aniline fuchsin made up as follows: Make a saturated solution of aniline oil in distilled water by shaking the two together. Filter and add 10 Gm. of acid fuchsin to 100 c.c. of the filtrate. The stain should be ready to use in about 24 hours. It deteriorates in a month. To stain, dry the slide with a towel, leaving the small area to which the section is attached, cover the section with stain, and heat over a small flame until strong aniline oil fumes come off; allow to cool; let the stain remain on the section about 6 minutes; return the stain to the bottle.

9. Absorb most of the stain with a towel and rinse in distilled water, so that the only remaining stain is in the section. If a large amount of stain is left, it will form a precipitate with methyl green. On the other hand, if too much stain is removed, the coloration of mitochondria will be faint.

10. Allow a little 1 per cent methyl green to flow over the section from a pipette, holding the slide over a piece of white paper, so that colors may be seen. Apply the methyl green for about 5 seconds at first and modify as required. This is the crucial point of the method.

11. Drain off excess of stain, plunge into 95 per cent alcohol for a few seconds, then rinse in absolute alcohol, clear in toluol, and mount in gum dammar.

Note: (a) The methyl green may remove all the fuchsin, even when applied for only a short time. This is due to incomplete mordanting of the mitochondria by the chrome salts in fixation. It may be avoided by omitting Steps 5 and 6 or by treating section with 2 per cent potassium bichromate for a few seconds just before staining. The purpose of potassium permanganate and oxalic acid is to remove the potassium bichromate.

(b) The fuchsin may stain so intensely that the methyl green removes it imperfectly or not at all. This is due to too much mordanting. It may be corrected by prolonging Steps 5 and 6.

(c) Sometimes, after obtaining a good differentiation, the methyl green is washed out before the slide is placed in toluol, in which event omit the 95 per cent alcohol and pass directly to absolute alcohol.

MYELIN SHEATH STAINS

Weigert-Pal Method

Weigert-Pal method is a slow, but very useful method.

See method on page 183.

Spielmeyer's Method

Spielmeyer's procedure is a very good stain to show fine detail of myelinated fibers.

See method on page 184 and Fig. 95 on page 185.

Courville-Krajan Method

Courville-Krajan method has many advantages of the longer methods and can be completed in 30 minutes.

See method on page 185.

Krajan's Myelin Sheath Stain

Krajan's myelin sheath stain has all the advantages of the longer methods, showing very fine detail of the fibrils, and can be completed in 1 hour.

See method on page 187 and Fig. 96 on page 187.

Pal-Kulschitzky's Method

Pal-Kulschitzky's method usually yields satisfactory results and is valuable for rapid staining of myelin sheath.

See method on page 188.

Morgan's Myelin Sheath Stain

Morgan's myelin sheath stain is a useful method to demonstrate myelin in paraffin sections.

See method on page 189.

NONMEDULLATED NERVE FIBERS

Ranson's Method

1. Fix segments of nerve for 48 hours in :

Alcohol, absolute	99 c.c.
Ammonium hydroxide, concentrated	1 c.c.

2. Rinse tissues in distilled water.
3. Place in pyridine for 24 hours.
4. Place in many changes of distilled water for 24 hours.
5. Place in 2 per cent silver nitrate solution for 3 days in a dark place.
6. Rinse in distilled water.

7. Reduce for from 24 to 48 hours in:

Pyrogallol -----	4 Gm.
Formaldehyde, 5 per cent, in distilled water -----	100 c.c.

8. Wash in water.

9. Dehydrate in graded alcohols, embed in paraffin, and cut sections.

The method is a modification of Cajal's technic for neurofibrils.

Nonmyelinated fibers appear black; myelinated axones, yellow; endoneurium, pale yellow.

NEUROGLIA FIBER STAINS**Cajal's Gold Chloride and Sublimate Method**

1. Fix tissues for from 2 to 10 days in the following solution:

Formaldehyde -----	15 c.c.
Ammonium bromide -----	2 Gm.
Distilled water -----	85 c.c.

2. Cut sections from 20 to 25 microns thick, collect, and place in distilled water to which a few drops of formaldehyde have been added. After quick wash, transfer 4 to 6 sections into separate glass dishes of about 6 cm. diameter, each containing 15 c.c. of:

Distilled water -----	60 c.c.
Bichloride of mercury -----	0.5 Gm.
Gold chloride, 1 per cent (Merck brown variety) -----	10 c.c.

3. After about 4 hours, the sections will have acquired an intense purple color, and are then placed for from 5 to 10 minutes in a fixing bath containing:

Sodium hyposulfite, concentrated solution -----	5 c.c.
Distilled water -----	70 c.c.
Alcohol, 95 per cent -----	30 c.c.
Sodium bisulfite, concentrated solution -----	5 c.c.

4. Wash in 50 per cent alcohol, transfer sections to slides, and blot in filter paper.

5. Dehydrate in 3 changes of absolute alcohol.

6. Clear in oil origanum for 5 minutes.

7. Wash in 2 changes of pure xylol. Mount in gum dammar.

Precautions.—Use pure brown gold chloride (Merck). If sections are left longer in the bath, good results may be obtained with or-

dinary gold chloride, but Cajal does not advise its use. Use only crystallized corrosive sublimate. Temperature must not be lower than 12° C. for human brain tissue; it should be between 18° and 22° C. For cerebellum, medulla, and spinal cord, between 22° and 26° C. For fixation, from 3 to 15 days is preferable, but fibrous neuroglia may be fixed up to a year. The material must be in fixative within 6 hours after death.

By this method of Cajal, neuroglia are stained a dark purple on a much lighter purple background.

Globus Modification of Cajal's Gold Sublimate Method for Neuroglia

Globus has provided a method of rendering tissue hardened in formaldehyde available for staining with gold chloride. This works out very well, provided the tissue has not been in formalin too long.

The following is his method:

1. Prepare frozen sections of formaldehyde-fixed material from 15 to 30 microns thick.
2. Wash quickly in several changes of distilled water.
3. Place in a 10 per cent solution of strong ammonia water for 24 hours at room temperature, or for a shorter period in an incubator.
4. Carry rapidly through 2 changes of distilled water.
5. Place in a 10 per cent solution of (41 per cent) hydrobromic acid and let it remain there for 2 to 4 hours.
6. Wash quickly in 2 changes of distilled water, to which a few drops of strong ammonia are added. Place in gold chloride solution:

Distilled water	-----	60	c.c.
Bichloride of mercury	-----	0.5	Gm.
Gold chloride, 1 per cent (Merek brown variety)	-----	10	c.c.

7. The sections assume an intense purple color after about 4 hours and are then placed for from 5 to 10 minutes in a fixing bath containing:

Sodium hyposulfite (concentrated solution)	-----	5	c.c.
Distilled water	-----	70	c.c.
Alcohol, 95 per cent	-----	30	c.c.
Sodium bisulfite	-----	5	c.c.

8. Wash in 50 per cent alcohol, float, and transfer sections to slides. Blot in filter paper.
9. Dehydrate in 3 changes of absolute alcohol.
10. Clear in oil origanum for 5 minutes.
11. Wash in 2 changes of pure xylol. Mount in gum dammar.

ASTROCYTES

Achucárro's Fourth Variant of Hortega

1. Fix tissues in 10 per cent formaldehyde.
2. Cut frozen sections and wash thoroughly in distilled water.
3. Place for 10 minutes between 45° and 50° C. in:

Tannin	3 Gm.
Ammonium bromide	1 Gm.
Distilled water	100 c.c.



Fig. 119.—Astrocytes in glioblastoma. Achucárro's method. Frozen section.
Line leads to Astrocytes.

4. Before the sections cool, wash in 20 c.c. distilled water containing 2 drops strong ammonium hydroxide, until they have regained their flexibility and transparency.

5. Place at once into dilute Bielschowsky's silver (1:10) until they assume a yellowish color.

Transfer sections to a fresh dish as soon as the liquid becomes turbid (use about 3 dishes).

6. Wash in distilled water.

7. Reduce in 20 per cent formaldehyde (neutralized with chalk).

8. Wash and dehydrate in absolute alcohol.

9. Clear in carbolxylol, wash in xylol, and mount in gum dammar.

Bielschowsky's silver solution, as recommended by Hortega:

Silver nitrate solution, 10 per cent	-----	30 c.c.
Strong ammonia	-----	40 drops

When made, it forms a precipitate. Wash the precipitate a dozen times with distilled water, using at least a liter of water. Add 50 c.c. distilled water to the washed precipitate, and then add ammonia drop by drop until it is dissolved. Avoid excess ammonia. Make it up to 150 c.c. with distilled water. Keep in a brown bottle in dark place.

This method demonstrates astrocytes.

GLIA FIBRILLA STAINS

Mallory's Phosphotungstic Acid Hematoxylin

Mallory's phosphotungstic acid hematoxylin is particularly useful for the demonstration of fibrin and neuroglia, fibroglia, and myoglia fibrils. It also brings out the detailed structure in mitotic figures, including the spindles and the centrosomes. See description of method on page 255.

Alzheimer Method

1. Tissues are fixed for at least 1 week, in 10 per cent formaldehyde. Frozen, paraffin, or celloidin sections may be used, but they should be cut 5 to 10 microns thick.

2. Mordant in:

Chromic acid, 1 per cent aqueous solution	-----	19 c.c.
Glacial acetic acid	-----	1 c.c.

3. Wash quickly but thoroughly in distilled water.

4. Place sections for 1 hour in paraffin oven at 55° C. in a saturated aqueous solution of acid fuchsin.

5. Wash in water until no more color comes off.
6. Agitate sections in a mixture of 30 c.c. saturated alcoholic picric acid solution and 60 c.c. distilled water.
7. Wash in 2 changes of distilled water.
8. Place sections in saturated aqueous solution of lichtgrün which has been diluted with an equal amount of distilled water for about $\frac{1}{2}$ hour.
9. Wash in water.
10. Dehydrate in 95 per cent alcohol, then in 2 changes of absolute alcohol.
11. Clear in 2 changes of pure xylol and mount in gum dammar. Glia fibrils stain bright red against a very pale green background.

Heidenhain Method

1. Fix tissues in 10 per cent formaldehyde.
2. Embed in paraffin in usual manner.
3. Cut sections 5 to 10 microns thick.
4. Remove paraffin by xylol and place in 2.5 per cent (violet) iron alum solution for from 6 to 12 hours.
5. Wash in distilled water rapidly but thoroughly.
6. Stain in 0.5 per cent (old) aqueous hematoxylin solution for a half hour or longer.
7. Wash in distilled water.
8. Differentiate in a weak solution of iron alum, controlling the differentiation under the microscope.
9. Wash thoroughly in tap water.
10. Rinse in distilled water.
11. Dehydrate in 95 per cent alcohol; follow with absolute alcohol.
12. Blot and clear in 2 changes of pure xylol.
13. Mount in gum dammar.

OLIGODENDROGLIA STAINS

Hortega's Silver Carbonate Method

1. Harden tissues for from 12 to 48 hours in formol-ammonium bromide solution:

Formaldehyde (Merck's neutral) -----	14 c.c.
Ammonium bromide -----	2 Gm.
Distilled water -----	86 c.c.

2. Bromurate. Heat blocks in fresh hardening solution for 10 minutes at 45° to 50° C.

Hydrobromic acid, 40 per cent	5 c.c.
Distilled water	95 c.c.

3. Cut frozen sections 15 to 20 microns.

4. Wash in 2 changes of distilled water. Add 10 drops of strong ammonia to the first water.

5. Impregnate. Place sections in strong silver carbonate solution and leave there 1 to 5 minutes:

Silver nitrate, 10 per cent	5 c.c.
Lithium carbonate, saturated solution	20 c.c.
Ammonium hydroxide, sufficient to dissolve precipitate	
Distilled water	75 c.c.

When the two chemicals are combined, silver carbonate comes down in a voluminous precipitate. The ammonia should then be added drop by drop, stirring all the while, until the precipitate disappears and ammoniacal silver goes into solution. A small amount of black dustlike precipitate remains undissolved and may be filtered off. The solution keeps well for a long time in a dark place.

6. Wash. Agitate gently for about 15 seconds.

7. Reduce by dipping sections into 1 per cent formaldehyde. Do not agitate.

8. Wash and tone in following solution until gray:

Gold chloride, yellow	1 Gm.
Distilled water	500 c.c.

9. Fix in 5 per cent sodium hyposulfite solution for 1 minute.

10. Wash in distilled water, dehydrate, clear, and mount in usual manner.

Oligodendroglia will stain selectively. There may be faint staining of astrocytes, and microglia may stain well.

Penfield's Modification of Hortega's Silver Carbonate Method

1. Harden tissues in ammonium bromide solution for from 2 to 48 hours. Blocks should not be more than 3 mm. in thickness. Place blocks in 95 per cent alcohol for from 36 to 48 hours.

2. Wash blocks about 4 hours (to eliminate alcohol) in several changes of distilled water in large volume.

3. Cut frozen sections. If alcohol is not sufficiently washed out, the cutting will be difficult.

4. Pass through 2 changes of distilled water.

5. Leave in strong silver carbonate (Hortega's silver carbonate) for from 15 minutes to 2 hours, as determined by trial. The most favorable time to remove sections is when they are just beginning to turn brown.

6. Plunge directly in 1 per cent formaldehyde and agitate at once.

7. Wash in water.

8. Tone in Hortega's gold chloride solution until gray.

9. Fix in 5 per cent sodium hyposulfite solution for 1 minute.

10. Wash, dehydrate, clear, and mount as usual.

This method, like the method of Hortega, is variable, although the results are brilliant when successful.

MICROGLIA STAIN

Hortega's Silver Carbonate Method for Microglia

1. Fix thin pieces of tissue, 2 or 3 mm., for 1 to 2 days at 35° C., or 2 to 4 days at room temperature:

Ammonium bromide	10 Gm.
Formaldehyde, commercial	70 c.c.
Distilled water	430 c.c.

2. Warm the pieces of tissue for 10 minutes between 50° and 55° C. in a fresh volume of the fixative.

3. Make frozen sections 25 to 30 microns thick.

4. Rinse quickly in 2 changes of distilled water, in which 2 drops of ammonia are added to each 50 c.c. of water.

5. Treat for 10 minutes between 15° and 20° C. in the following silver carbonate solution:

Silver nitrate, 10 per cent	5 c.c.
Sodium carbonate, 5 per cent	20 c.c.

Add ammonia drop by drop until the yellow precipitate is just dissolved, then add 15 c.c. of distilled water.

6. Reduce in a 10 per cent solution of acid-free formaldehyde. Move sections during this process. A simple expedient is to place them in a large Petri dish and blow on the fluid gently.

7. Wash in distilled water.
8. Tone in a 0.2 per cent solution of yellow gold chloride, until the sections are gray (it usually takes 10 to 15 minutes).
9. Treat in a 5 per cent sodium hyposulfite solution for 1 minute.
10. Dehydrate in 95 per cent alcohol.
11. Clear in carbolxylol-creosote mixture:

Phenol -----	10 Gm.
Xylol -----	80 c.c.
Creosote, pure -----	10 c.c.

12. Mount in gum dammar.

Microglia stain deeply; fibrous neuroglia and glia fibers stain faintly.

OLIGODENDROGLIA AND MICROGLIA STAIN

Penfield's Combined Method

1. Harden tissue in 10 per cent formaldehyde or formol-ammonium bromide for an indefinite period. About a week's fixation in formaldehyde gives excellent results.
2. Cut frozen sections 20 microns thick, and place them in 1 per cent formaldehyde or distilled water. Through the succeeding steps, handle sections with a glass rod.
3. Place in a dish of distilled water to which 10 to 15 drops of strong ammonium hydroxide have been added, and cover so as to prevent escape of ammonia. Leave in this solution overnight to remove formaldehyde.
4. Transfer directly to Globus hydrobromic acid in 5 per cent solution (5 c.c. of 40 per cent hydrobromic acid to 95 c.c. of distilled water). Place in incubator at 38° C. for 1 hour.
5. Wash in 3 changes of water.
6. Place in a 5 per cent solution of sodium carbonate for 1 hour. Sections may remain in this solution for from 3 to 6 hours without ill effect.
7. Pass with or without washing directly into del Rio Hortega's silver carbonate, weak solution, and leave there for from 3 to 5 minutes. Sometimes they may be left until they begin to turn yellowish gray. Then transfer to reducer. Control the duration in silver solution by taking out a section at intervals of a minute or two and examining under the microscope. The sections should turn a smooth gray color in the reducer (formaldehyde).

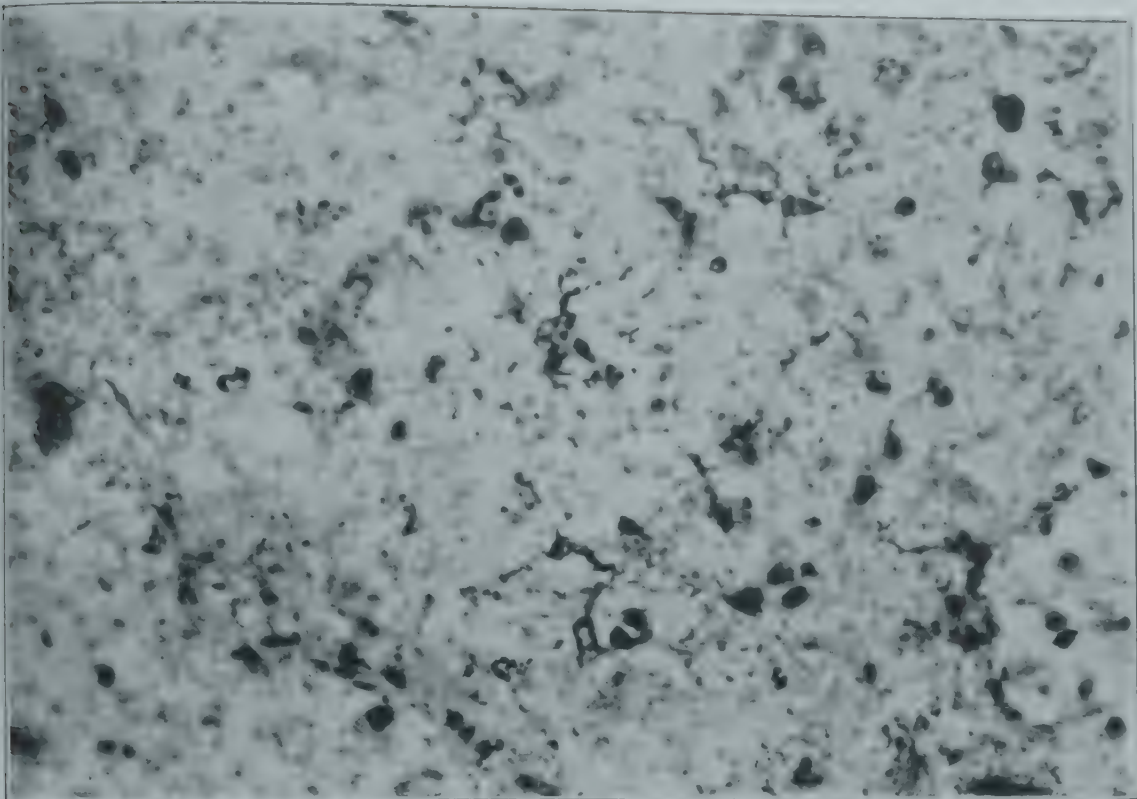


Fig. 120.—Early changes of microglia in old gunshot wound of the brain. Penfield's combined method. Frozen section.

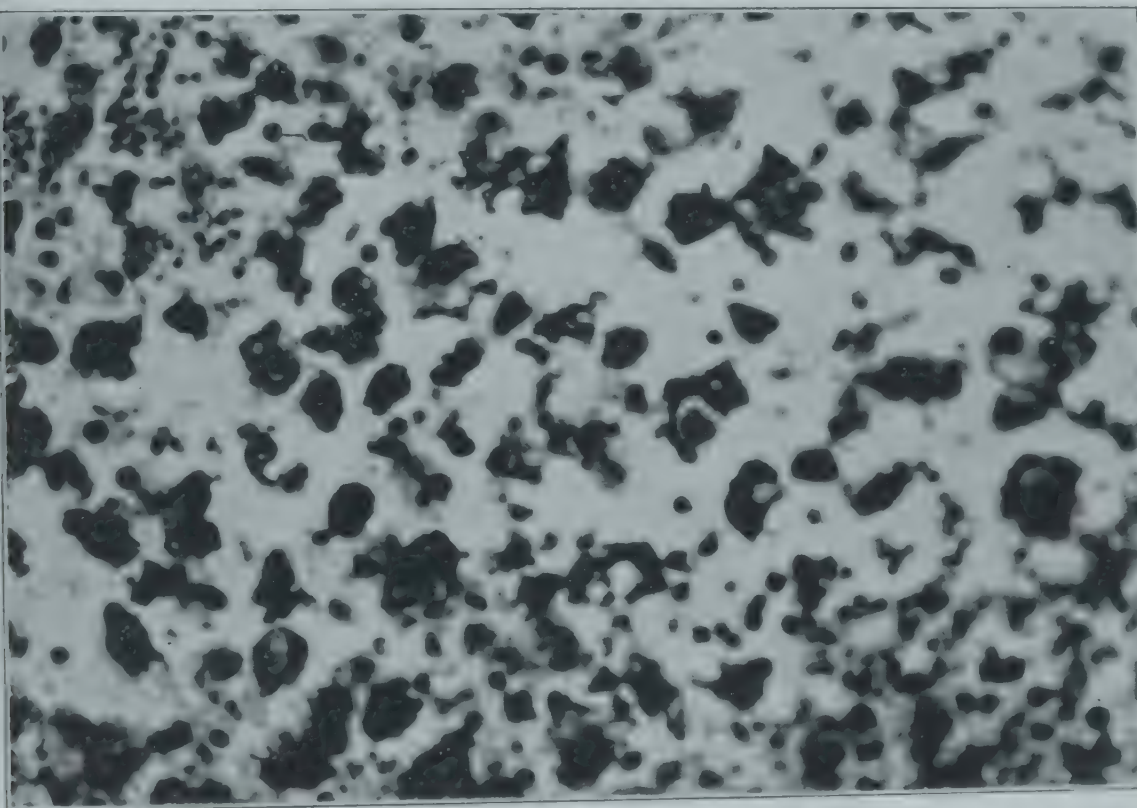


Fig. 121.—Microglia developing phagocytes at the margin of a brain wound. Penfield's combined method. Frozen section.

8. Plunge in 1 per cent formaldehyde and agitate.
 9. Wash in distilled water.
 10. Tone in gold chloride (1:500) at room temperature until a yellow tint disappears and the sections are a smooth bluish gray.
 11. Treat in 5 per cent sodium hyposulfite.
 12. Wash in distilled water.
 13. Float onto slides and flatten out with needle. Dehydrate in 2 to 4 changes of 95 per cent alcohol from a drop bottle. Follow this with a few drops of carbolxylol. When clear, drain the slides and blot immediately with 2 thicknesses of fine filter paper.
- Mount in gum dammar.

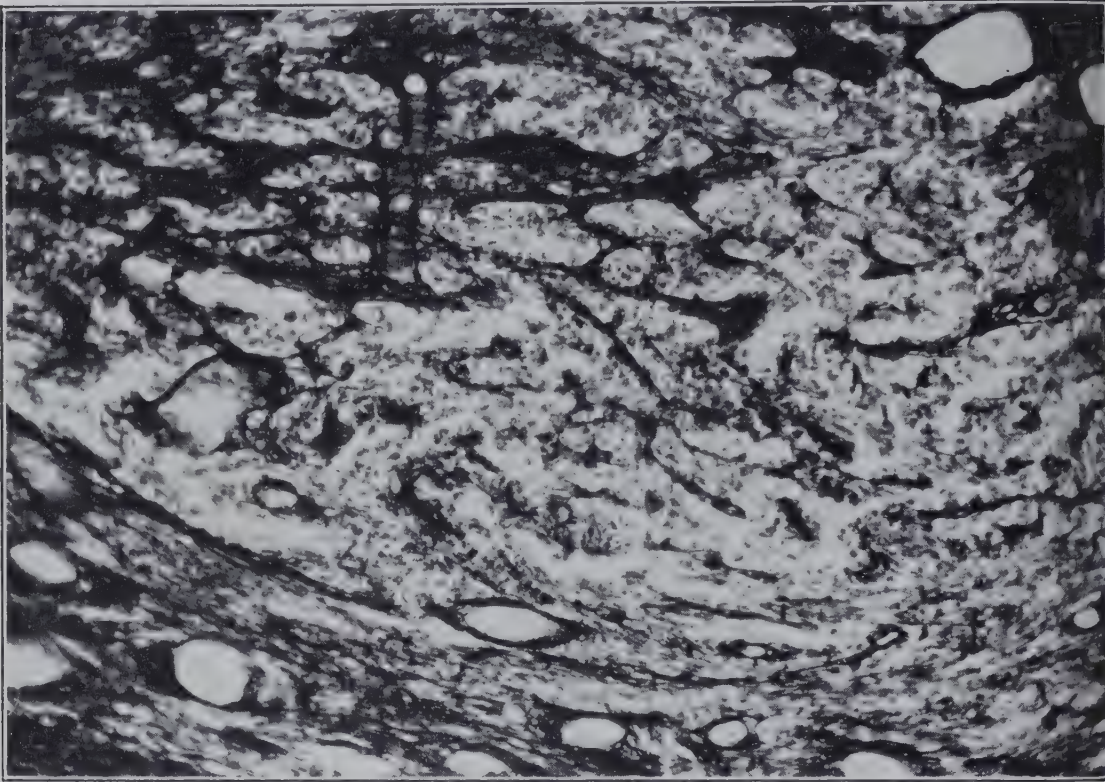


Fig. 122.—Reticulum in a ganglion cell tumor. Krajan's reticulum stain. Reticular fibers stain black. Frozen section.

Del Rio Hortega's ammoniacal silver carbonate (weak solution):

Silver nitrate (Merek), 10 per cent solution	-----	5 c.c.
Sodium carbonate (pure), 5 per cent solution	-----	20 c.c.
Ammonium hydroxide—sufficient to dissolve the precipitate		
Distilled water to make	-----	75 c.c.

The ammonium hydroxide in strong fresh solution should be added drop by drop until the precipitate is just dissolved, stirring

the solution all the while. It is important not to add too much ammonia. A fine black sediment should remain behind which does not resemble the more voluminous precipitate of silver carbonate. This fine sediment should be filtered off. The solution may then be preserved in a dark bottle for long periods.

The yellow variety of gold chloride is less expensive than the brown and seems to be preferable for toning.

This method of Penfield's is highly recommended.

RETICULUM STAINS

Perdrau's Modification of Bielschowsky Method

Perdrau's modification of the Bielschowsky method is a good but slow method of staining reticulum. See method on page 154.

Krajian's Rapid Method for Staining Reticulum and Collagen Fibers in Frozen Sections

An excellent and rapid method of staining reticulum is discussed on page 154.

Foot's Method for Staining Reticulum in Paraffin Sections

Foot's method for staining reticulum is a good and rapid method for paraffin sections. See method on page 156.

CONNECTIVE TISSUE STAINS

Mallory's Aniline Blue Stain

(Modified)

Mallory's aniline blue stain (modified) is an excellent method for connective tissue fibers. See method on page 147.

Van Gieson Stain

(Modified)

Van Gieson stain (modified) is a rapid and useful method to demonstrate collagen fibrils. See method on page 152.

HEIDENHAIN'S AZOCARMINE STAIN

Heidenhain's azocarmine stain is an excellent method to demonstrate the minute details of connective tissue.

See method on page 148.

FAT STAINS

Scharlach R

Originally, sudan III was used to stain fats, but the staining reaction is less brilliant than scharlach R which is now preferred. The solution is prepared by saturating the dye in equal parts of 7 per cent alcohol and pure acetone. The staining takes place in a few minutes. Neutral fats stain dark red.

See description of method on page 176.

Nile Blue Sulfate

(Lorraine Smith)

Nile blue sulfate is not as general a stain for fat as scharlach R but it has certain valuable properties. It gives a double stain coloring neutral fats red, cholesterin ester and cholesterin-fatty acid mixtures reddish, and fatty acids and soaps deep blue.

See description of method on page 178.

Osmic Acid

Tissue is stained in the block, using osmic acid either in simple solution or in special formula, such as Marchi's. Blocks are embedded in paraffin, and thin sections are cut. Great care should be taken not to use fat solvents in the process of staining and embedding. Better results are obtained by using single frozen sections of formaldehyde-fixed tissue, rather than whole blocks.

See description of method on page 177.

MUCIN STAIN

Mayer's Mucicarmin Stain

Mayer's mucicarmin stain is a specific staining method for the demonstration of mucin in tissue sections. Mucin stains red.

See description of technic on page 174.

MELANIN STAIN

Masson's Silver Method

By Masson's method the melanin pigments stain brown black. See description of method on page 239.

IRON STAIN

With Potassium Ferrocyanide

By this method hemosiderin pigments stain intensely blue, even to the finest granules.

See description of method on page 238.

SPIROCHETE STAINS

Levaditi and Manouelian Methods

For the demonstration of *Spirochaeta pallida*, the Levaditi method has been the standard technic for many years. More rapid and improved methods have been devised which are far superior to it.

See description of method on page 220.

Dieterle's Method

By Dieterle's method excellent results can be obtained within 6 hours.

See description of method on page 221.

Krajian's Rapid Method

Krajian's method can be completed, with excellent results, within 20 minutes.

See description of method on page 224.

ELASTIC TISSUE STAINS

Weigert's Method

This is excellent for elastic tissue. See method on page 131.

Krajian's New Elastic Tissue Method

A rapid method to demonstrate elastic fibers in frozen sections. See method on page 132.

MacCallum's Modification of Verhoeff's Method

An excellent method of staining elastic fibers in paraffin and frozen sections. See method on page 132.

PART XI

MISCELLANEOUS METHODS

PREPARATION OF MUSEUM SPECIMENS

The preparation of museum specimens is a very important part of laboratory technic. Hospitals should each develop a medical museum in order to preserve valuable and unusual specimens for scientific and, at times, medicolegal purposes.

Information gathered in a course given by Dr. E. L. Judah was largely used in the compilation of this chapter and we wish to express our indebtedness to Dr. Judah for the fine instruction given by him in his very efficient department at McGill University.

Solutions for the Preservation of Specimens Kaiserling Method

The Kaiserling method gives by far the best results for ordinary pathological and anatomical preparations where the color of the red blood cells is to be preserved. If any of the faint yellows or greens (due to bile staining or other causes) are to be kept, one of the chloral hydrate fluids, preferably Klotz, gives excellent results, with Kaiserling No. III as a permanent preservative.

Specimens must be obtained fresh from the autopsy or operation and not allowed to soak in water for more than a few moments. Only the superfluous blood should be washed away, remembering that the one color available for preservation lies in the hemoglobin of the red blood corpuscle, and that this, therefore, must be preserved. The basis of the methods of color preservation which are to be recommended lies in the chemical change produced by the action of formaldehyde upon the hemoglobin of the red cell and its subsequent fixation as the red alkaline hematin by alcohol.

It is necessary to enlarge on the proper laying out of the specimens in the No. I solution. No cloth, metal pins, nor paper must be allowed to come in contact with them. Cotton wool to hold in shape and stretching on glass frames with cotton thread is all that is necessary. Large bodies of fresh fluids must be used. After care-

ful comparison both abroad and at home, the following process has been found to give the best results:

Kaiserling Solution No. 1

Potassium acetate -----	170 Gm.
Potassium nitrate -----	90 Gm.
40 per cent formaldehyde (Formalin*) -----	1,600 c.c.
Water -----	8,000 c.c.

Care should be taken to use only high-grade chemicals.

1. Water white formaldehyde must be as free from formic acid as possible. It is not necessary to buy the chemically pure grade. A white deposit, due to freezing (paraformaldehyde) is sometimes seen in the Formalin. This lessens the concentration of formaldehyde gas, and consequently the color and fixing processes are not correct. Always buy Formalin in the spring of the year and see that there is no white deposit present. Do not boil it unless you restore the amount of Formalin used in making the solution.

If the formaldehyde is found to be acid, it can be neutralized with sodium carbonate or sodium hydroxide.

To make a 2 per cent Formalin solution:

Formalin -----	5 parts
Water -----	95 parts

4 per cent: Use 10 parts of Formalin to 90 parts of water.

6 per cent: Use 15 parts of Formalin to 85 parts of water.

2. Potassium acetate. We recommend Mallinckrodt potassium acetate.

3. Potassium nitrate. Merck's pure powder, U.S.P., is excellent. (It is not necessary to use C.P.)

4. Glycerin, water white (C.P.). Do not use dynamite glycerin as it produces a change in color.

5. Chloral hydrate.

The length of time the specimen should remain in Kaiserling's first fixation fluid has caused a great deal of comment. It is of secondary importance to thorough hardening. The specimen should be fixed in such way that no blood will ooze out when firmly pressed between the fingers. In fact, good color results have been obtained in specimens that have stood from 2 to 4 months in the No. 1 solution. These are, of course, exceptional cases. Intestines

*Water white formaldehyde.

should not be left for more than 10 to 12 hours. Other specimens or organs should be left in the solution from 1 to 5 days. In all instances they must be thoroughly fixed. Formalin increases the firmness of tissue. The tissue loses its natural color and assumes an aged and grayish appearance.

Great care must be taken to wash the specimens thoroughly in running water after removal from this solution.

Kaiserling No. II

Ninety-five per cent alcohol is used. It may be used at once without the intermediate stage of 80 per cent alcohol recommended by Kaiserling. The specimens must be left here until the natural colors, which have disappeared under the action of the formaldehyde in the first solution, return, the process being watched in the same way as in the development of a photographic plate.

The strength of the alcohol should not be allowed to go below 80 per cent. To avoid this the specimens should be transferred to a second alcohol of the original strength. The time in which the specimens remain in the alcohol varies according to the particular character of the tissue.

In this stage again the specimens must be carefully washed in water, no traces of the formaldehyde or alcohol used in the first and second solutions are to be carried to the final fluid. If either the alcohol or the Kaiserling or the Kaiserling No. III solution used gives off any odor of either formaldehyde or alcohol, it must be thrown away. This is absolutely necessary.

When specimens have been left in alcohol for too long a time, color can be restored by leaving them in equal parts of Kaiserling No. III and glycerin for 24 hours, allowing for the amount of glycerin already in the Kaiserling fluid.

Kaiserling No. III

Potassium acetate -----	4 lb.
Glycerin -----	2,000 to 4,000 c.c.
Distilled water -----	10,000 c.c.
to which 20 c.c. carbolic acid may be added as a preservative.	

In preserving veterinary or comparative anatomy specimens Kaiserling is not satisfactory: the best results have been obtained by Klotz, increasing the Formalin possibly to 6 per cent, then placing in the alcohol, and preserving in Kaiserling III.

When specimens in Kaiserling III in open jars are used to pass around a class, care must be taken not to allow the surface to dry, or the fluid to be contaminated with molds. One infected jar may cause contaminations in many others and necessitate putting them through No. I and No. II solutions just as you would with a fresh specimen.

To Clarify Kaiserling Solutions

To get a clear Kaiserling No. III, connect a five-gallon vessel with an air compressor. Run the Kaiserling through a Berkefeld filter. This takes 20 to 30 minutes. Run water through the filter to clean it. Be sure to connect the filter with the water main.

Another method of clarifying Kaiserling No. III follows:

For 10,000 c.c. of Kaiserling No. III, 5 funnels are required. Prepare filter paper. Do not fold it. Place the 5 filters in a row. Use 1 tablespoonful of charcoal (very fine bone charcoal) for each funnelful. Put the charcoal in a jar and shake well with Kaiserling No. III. Run the solution through the funnels slowly. Refilter the first filtrate that comes through the funnels.

In making Kaiserling No. III, do not use tap water. Use distilled water; tap water contaminates the solution.

Kaiserling No. III dissolves plaster of Paris.

Klotz formerly used a little formaldehyde in Kaiserling No. III. This is not recommended as it causes an undesirable chemical reaction. He has now changed to carbolic acid. Carbolic acid makes a specimen slightly gray but prevents the growth of fungi.

Important points to remember are: There must be no odor of formaldehyde or alcohol in Kaiserling No. III. Wash out both of these materials thoroughly so that no trace of odor remains. Overharden in Kaiserling No. I rather than underharden. Always sacrifice color to permanency. Avoid infecting the jars. If infected, specimen must be treated again with Kaiserling Nos. I, II, and III.

Formalin will not penetrate either liver or lungs. In order to fix and preserve color, they must either be cut into slabs or injected.

For red blood cell color preservation, place the specimen in a Novy jar and cover with Kaiserling No. I. Then saturate with carbon monoxide gas from any gas jet. To eliminate excess gas, burn off the exhaust or connect a tube to the Novy jar and extend it out of the window.

Always protect a specimen from the direct rays of the sun even after it is completed. Colors are affected by sunlight. Diffuse sunlight will not affect them. Heat will destroy color. Keep specimens in a cool place.

Never preserve a specimen in pure glycerin. A spleen preserved in pure glycerin will shrink to one-half its size.

Be careful of the concentration of alcohol in Kaiserling No. 11 because it will lose its strength. Keep above 80 per cent. Use a small alcoholometer employing a small test tube (Arthur H. Thomas Company).

Frost's Sugar Solution

Sodium fluoride -----	80 Gm.
Chloral hydrate -----	80 Gm.
Potassium acetate -----	160 Gm.
Cane sugar (Tate's cubes) -----	3,500 Gm.
Saturated thymol water -----	8,000 c.c.

Sodium fluoride may be omitted. Tate's cube is not necessary. Use cane sugar. Never, under any circumstances, use beet sugar.

Method of Klotz and Jores

Prepare artificial Carlsbad salts in the order given below, and add to fixing solution:

Artificial Carlsbad Salts

(Klotz and MacLachlan)

Sodium sulfate -----	11 Gm.
Sodium bicarbonate -----	10 Gm.
Sodium chloride -----	9 Gm.
Potassium nitrate -----	19 Gm.
Potassium sulfate -----	1 Gm.

1. Fix specimens for from 1 to 5 days or longer in the following fixing solution:

Artificial Carlsbad salts (above) -----	50 Gm.
Chloral hydrate -----	50 Gm.
Formaldehyde, commercial -----	100 c.c.
Water -----	1,000 c.c.

2. Wash in running water for 24 hours to remove traces of chemicals and formaldehyde.

3. Drain and preserve in the following solution :

Potassium acetate -----	300 Gm.
Glycerin -----	400 c.c.
Saturated arsenious acid water -----	1,000 c.c.

Saturated arsenious acid is made by boiling an excess of arsenic trioxide in water for 2 hours. Allow to cool, and filter or decant off clear fluid.

Steps in the Preparation of Museum Specimens

1. Place the specimen through Kaiserling solution No. I until the specimen is thoroughly fixed. Wash thoroughly in water.
2. Put the specimen in Kaiserling No. II until the color is restored. Wash thoroughly in water.
3. Put the specimen in Kaiserling No. III. This is the final preservative.
4. Dissect the specimen and prepare it for the museum jar.
5. Select a jar of proper size. Use the smallest museum jar that will hold the framed specimen.
6. Make a frame for the museum jar.
7. Bore a hole in the glass cover of the jar.
8. Stitch the specimen on the frame but do not make the final knot in tying the specimen. Use a single surgeon's knot.
9. Place the specimen in the jar and cover with Kaiserling No. III.
10. Let stand overnight.
11. Make a paraffin tag and sew it onto the specimen.
12. The following day, adjust the specimen to the frame; observe for proper balance and centering.
13. Pour out the Kaiserling No. III solution.
14. Tie final knots, this time tying a surgeon's knot over the original surgeon's knot.
15. Trim off the knots and move them to the back of the specimen, using a pair of forceps.
16. Wash the specimen in cold water. Cover the specimen with cold gelatin and let it drain. (Use Muir's solution.)
17. While the specimen is draining, wash the jar with soft soap.
18. Be sure the hole in the cover of the museum jar is the correct size.
19. Place the specimen in the jar; be sure that the front of the specimen is to the front of the jar.

20. Fill jar with Kaiserling No. III up to within one inch of the top.
21. Seal the cover on the jar.
22. Let stand overnight.
23. Clean off excess cement with gasoline.
24. Fill the jar with Kaiserling No. III. This is done through the hole in the cover.
25. Place a small cork in the hole in the lid of the jar.
26. Seal with cement.
27. Place a card holder on the top of the jar, using cement, and put card label in place.

Museum Jars

Buy museum jars direct from the jobbers. Buy practically all narrow jars (not square) to bring the specimen up as close to the jar as possible. The proper-sized jar will save fluid, is handled easily, and will save shelf space in the museum rooms. Use small jars when possible. In addition to the odd sizes of jars, fixation jars are used. There are six or seven sizes required. These do not have to have polished surfaces as they are viewed from the top and not from the sides. They are used for preservation and storage.

Be careful to give specifications when ordering. Specify jars (a) without foot; (b) without lower border; (c) without projecting upper rim.

The lids are ground to fit the jars. The front surface of the jar is planed and polished to remove any irregularities. The entire front surface should be planed carefully because if a tiny spot on the edge is left, it will throw shadows. Return defective jars or those which do not meet the specifications. Well-made jars are not blown in iron molds. Metal chills faster than wood; consequently, the sides contract inward and the corners are too thin. Unpolished jars are always cast in iron molds. Polished jars cost 35 per cent more than unpolished jars but must be used. You can purchase special jars. It is best to order run-of-the-mold jars.

There are certain variations in manufacture, some to be expected but some absolutely undesirable. Some jars have slightly uneven bottoms. Some have a few faint waves on the sides or back. Some have bubbles in them. If the bubbles do not interfere with the view of the specimen, such jars can be used. Otherwise, return the jars. Covers that protrude slightly over the edge of the jar can be ground

down on stone. If you cannot grind them down, take them to an optical company and have them ground.

Return to the manufacturer (1) jars with defects that interfere with the view of the specimen; (2) jars with wall too thin to allow proper sealing; (3) jars with covers that do not fit flat or properly.

Do not expect perfect jars as the cost is prohibitive and the others are just as good. Those planed and polished on both sides are not necessary. Round jars are undesirable because they have many defects, which cause them to break. In handling round jars, pick them up from the bottom, not from the clamp or handle as the jar usually slips and breaks if mishandled.

Never make your own jars. It has been found unprofitable. For storage, round jars that taper toward the bottom are convenient but are almost impossible to open once they have been sealed. Mason jars are abominable for storage. The fluid usually runs over the sides and you have to break them to get the specimen out.

We suggest Whitehall-Tatum jars for storage. Covers for storage jars must fit inside the jar, not over the sides.

Crocks from Arthur H. Thomas Company (made by Red Wing Manufacturing Company of Red Wing, Minn.) are good. Terra cotta is not recommended for museum work. Specify felspar glaze when ordering crocks.

Glass-stoppered jars are not recommended. Too much space at the top is lost and the stoppers stick when the temperature changes.

Good jars may be obtained from Ingram and Bell and Hughes-Owen of Montreal, or from Clay-Adams Company, New York City.

Specifications

These jars are made without foot or lower border or projecting upper rim, the front surface being planed and polished. Each jar is supplied with a plain glass cover, the lower border of which is ground to meet the upper border of the jar which is also ground. A small 3 mm. hole is bored in the lefthand back corner of the cover through which the jar is filled when sealed. It also acts as a safety valve to allow for expansion and contraction of the fluid.

Dissection

Dissection is always made under a fluid. Never tease out anything. Always cut off clean with a pair of scissors. It is important to work under water because you can see in this way whether the

Inside centimeter measurement

<i>Heart Jars</i>				
No.	1.	19	× 19	× 13
	2.	19	× 13	× 13
	3.	19	× 13	× 10
	4.	17	× 11	× 11
	5.	14½	× 12½	× 9½
	6.	14½	× 12½	× 8
	7.	13	× 12	× 9
	8.	12	× 10½	× 7½
	9.	10	× 6	× 9
	10.	10	× 5	× 4
	11.	8	× 4	× 3

<i>General Utility Jars</i>				
No.	16.	40	× 40	× 20
	17.	35	× 35	× 15
	18.	30	× 30	× 12
	19.	28	× 26	× 12
	20.	25	× 21	× 10½
	21.	22	× 19	× 9
	22.	22	× 16	× 9½
	23.	20	× 17	× 7
	24.	19	× 14	× 6½
	25.	18	× 13	× 5½ <i>fairly thick</i>
	26.	17	× 12	× 5
	27.	16	× 11	× 4
	28.	16	× 10½	× 3
	29.	15	× 10	× 2
	30.	14	× 9	× 3
	31.	14	× 9	× 2½ <i>do not use many</i>
	32.	14	× 8	× 2
	33.	13	× 9	× 4
	34.	12	× 9½	× 3
	35.	12	× 8	× 2
	36.	11	× 10	× 4½
	37.	11	× 10	× 3½
	38.	11	× 9	× 2½
	39.	10	× 8	× 3
	40.	10	× 7	× 2
	41.	8	× 8	× 3
	42.	7	× 8	× 2½
	43.	8	× 7	× 2½
	44.	19	× 6	× 4
	45.	18	× 5	× 3½
	46.	17	× 1	× 3

<i>Kidney Jars</i>				
No.	105.	17	× 17	× 6 <i>Use many of these.</i>
	106.	16	× 16	× 5
	107.	13	× 14	× 4

<i>Fetus Jars</i>				
No.	110.	28	× 12	× 10½
	111.	26	× 11	× 8
	112.	22	× 10	× 8 <i>Amputations, monstrosities, etc.</i>
	113.	22	× 9	× 7
	114.	19	× 9	× 9
	115.	17	× 7	× 5
	116.	15	× 6½	× 4
	117.	13	× 6	× 4

<i>Intestine and Artery Jars</i>				
No.	50.	40	× 19	× 10
	51.	55	× 19	× 7
	52.	30	× 15	× 6
	53.	25	× 15	× 5
	54.	22	× 15	× 4½
	55.	20	× 14	× 4
	56.	35	× 9	× 5
	57.	32	× 9	× 5
	58.	29	× 9	× 4
	59.	28	× 8	× 4
	60.	25	× 7	× 3½
	61.	21	× 7	× 3
	62.	18	× 7	× 3
	63.	18	× 6	× 2
	64.	18	× 4	× 1½
	65.	16	× 4	× 1½
	66.	14	× 4	× 2
	67.	12	× 4	× 1½
	68.	10	× 4	× 1½
	69.	8	× 4	× 1½
	70.	6	× 4	× 1½
	71.	10	× 2	× 1
	72.	8	× 2	× 1

Can cement them on plate glass with slight bevel.

<i>Stomach and Uterus Jars</i>				
No.	85.	19	× 27	× 12
	86.	19	× 26	× 8
	87.	17	× 22	× 10
	88.	16	× 22	× 8
	89.	15	× 20	× 6
	90.	12	× 20	× 6
	91.	11	× 15	× 3
	92.	10	× 13	× 3
	93.	8	× 9	× 2½

Use a lot of these.

<i>Placenta Jars</i>				
No.	95.	28	× 26	× 9
	96.	26	× 26	× 8
	97.	24	× 24	× 7
	98.	22	× 22	× 6
	99.	21	× 21	× 5
	100.	20	× 20	× 4

Can use for lung slabs. Most convenient.

<i>Eye Jars</i>				
No.	120.	5	× 5	× 3
	121.	5	× 5	× 2½
	122.	4	× 4	× 2
	123.	4	× 3	× 1½

Not planed and polished.

Have to keep in stock. Can use for small specimens.

<i>Fixation Dishes</i>				
A.	30	× 50	× 35	
B.	25	× 45	× 30	
C.	25	× 40	× 25	
D.	20	× 35	× 20	
E.	8	× 35	× 25	
F.	5	× 33	× 23	
G.	8	× 25	× 16½	
H.	5	× 23	× 16	

Want clear glass but not necessarily water white. No covers bored.

specimen looks "cottony." Do not leave any fluff. Dissect out all undesirable material before mounting. Do not under any circumstances use a scalpel.

To Make Glass Frames

Pathological specimens should be placed in jars so that the specimen will be in its correct anatomical position. For this purpose, use the smallest jar that will fit it correctly; one that will hold the smallest amount of fluid should be used. In this way one saves museum space and fluid.

Precautions.—

1. The glass rod should be dry before flaming. Dry with cloth or alcohol if it is wet. Wait until alcohol is dry before beginning to work.

2. Do not wash jar until after the frame is made. Be sure jar is not cracked before making frame for it.

3. Do not try other methods of making frames until this one is mastered.

4. We suggest the use of soda glass rods. It is not necessary to buy standard glass. Glass rodding in sizes from 2 to 10 mm. may be used. Do not purchase dry glass, as it is very hard to work. Thermometer glass is useless.

Technic.—

1. Adjust a blowtorch.

2. Mark the glass the size of the base of the jar, starting from the left hand front corner of the jar, marking the rod so that it is shorter than the base of the jar by just the diameter of the glass rod.

3. Heat until "bent" at a right angle. Right angle is "bent" by placing the hot glass across the top of the jar until it just fits. This must be done while the glass is still hot. Place in the bottom of the jar when molten to take the shape of the bottom of the jar.

4. When set, measure up to bend *C*, and mark. (See Fig. 125.)

5. Heat, and bend at *C*. While still hot, place the jar on its side, and lay the hot glass over it. Flatten out.

6. Mark, and bend to *D*.

7. This brings point *E*, or the very end of the rod, on the outside of point *A*. File off.

8. Measure to see if it fits the jar. If it is a trifle too long, file, and reheat *D* and pull down.

9. Heat *E* and *A* until they fuse. Heat *C* to take care of expansion of the glass.

10. Measure the inside diameter of the jar from front to back, and cut two pieces of glass rod of this size. These are shorter than the top diameter of the jar, and consequently must be tested to see if they fit in the bottom.

11. Cut one piece so that it fits the top diameter of the jar.

12. Round off the edges of the rod in the flame. (There are now three pieces of rod called the feet.)



Fig. 123.—Blowtorch.

(Figs. 123 to 131 from Gradwohl: Clinical Laboratory Methods and Diagnosis.)

13. Apply the feet to the glass frame. Hold the frame in the left hand. Place the foot on a soapstone. Place the glass frame over it. Place the feet according to the position desired for the front of the specimen. If the specimen is to go in the direct center, the frame is placed in the center of the feet.

Apply the torch at the point where the feet and the frame touch, until they fuse. Then immediately place into the jar and press

down. This must be done while the glass rod is still in the molten condition. Then apply the other foot. One foot at a time is placed on the frame. Applying the first leg is the most difficult. Put the left leg on first.

14. Place the top rod on the glass frame employing the same technic as 13. above.

15. Frame must just fit into the jar, with room at the top for the threads. Cover must fit on the jar after the feet are in position, or it will not seal.



Fig. 124.—Bellows.

16. Heat one corner of the frame red-hot to allow for expansion and contraction.

“Pointers” on Making Frames. The greatest difficulty is in applying the first leg. Put on at point A. Adjust by laying flat on soapstone and setting.

Remember that the frame must just fit, and no more.

Sufficient space must be left at the top for threads. These go over the glass frame to hold the specimen in place.

It is necessary that the frame fit across the bottom of the jar so that the weight of the specimen will not be on the legs. These often break if this point is not observed. By placing the glass frame in the molten condition in the jar, and allowing it to set, this difficulty can be overcome.

When the glass frame cools, the surface contracts. The contraction must be allowed for by reheating one corner, red-hot.

Fuse the ends of the glass feet so that the specimen will look well in the jar.

For square jars, turn the ends of the glass feet directly into the corners of the jar. These feet are called *arrows*.

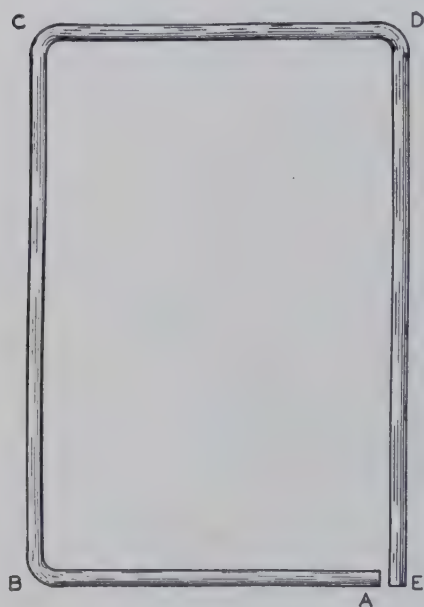


Fig. 125.—Bending glass frame.



Fig. 126.—Completed glass frame.

For round jars, curve wire around the specimen. Put an eye at each end. Then make a glass curve like it. Put it around the specimen to hold its natural curve. Use 2 mm. glass rod.

In heating colored glass, approach the flame carefully. There is arsenic in the coloring process and the glass is likely to shatter. Colored glass is obtainable from Central Scientific Company, Chicago, or E. H. Sargent Company, Chicago.

Care must be taken in making frames for specimens without fluid. They must be well made and very, very firm in structure.

Stitching Specimens Into Frames

All specimens must be held securely in their frames:

Tie the specimen into its frame by using two threads at the top, two threads at the bottom, and two threads on each side. This will hold the specimen rigid. Be sure that opposite stitches are in line, as irregular stitches change the appearance of the specimen. Leave long ends on these threads. Stitches are put in tight, not taut. Spread stitches so that the pull is toward the corner of the frame, not the middle, as there is more strength at the end of the frame than in the center. You may vary this occasionally. Never begin the stitches at the center of the specimen.



Fig. 127.—Heart mounted, ready for the museum.

Never use heavier thread than is absolutely necessary. No. 50 is used more than any other, although it is very fine. Nos. 10, 20, 30 are often used. You can occasionally use black thread.

Use a double surgeon's knot. Take two turns of the thread. The next day when tightening, take two more turns and tie evenly. After the knots are tied, bring them to the back of the specimen by means of a pair of forceps. This conceals the knot. Then clip the ends off with scissors. Before proceeding to this final step of tying

the specimen, place the specimen in the jar with the knots (surgical) over the glass frame, and allow it to remain overnight, covered with Kaiserling No. III. This allows for shrinkage of the specimen and the threads. If the knots are tied too tightly, the glass frame will break when shrinkage occurs. The tying of the knots is the *last step* before sealing the jars. For complete technic of preparation of specimens, see page 327.

To Mount a Brain.—Draw the ends of two pieces of glass rod out to a point. They must be longer than the specimen for which they are to be used. Pierce directly through the upper part of the brain with the pointed glass rod. Pierce directly through the lower part of the brain in the same fashion. Cut off the ends of the glass rod even with the brain tissue at all four ends. This conceals the glass rod inside the brain tissue. To mount the brain on its glass frame, use an upholsterer's needle with a long thread. Push it through the brain diagonally so as to surround the second rod with thread. Do not plan to surround the rod nearest to the point of entrance. Draw the thread through the brain and bring out on the other side. Enter the brain again diagonally, surrounding the second rod and bringing the needle and thread through the brain. When this thread is drawn up through the brain tissue, it will be anchored around the glass rod. Do the same thing in the opposite direction. Tie the ends of thread to the glass frame after centering the specimen.

For Soft Tissue That Will Tear.—Use fine glass tubing. First, go through the tissue with a suture. Put one hole at the top of the specimen, and one at the bottom of the specimen. Take a piece of glass tubing, and round the end in the flame, but be careful that the holes do not close. Follow the suture through with the glass tubing. Cut the tubing off at both ends, permitting it to extend beyond the tissue for a short distance.

Put a long upholsterer's needle through the glass tubing. The needle contains four strands of thread. Repeat at the bottom tubing. With two strands of thread tie the specimen to the top of the frame. With the other two, tie to the upper sides of the frame. From the lower tubing, tie two strands of the thread to the lower part of the frame, and tie the other two threads on either side. This gives two threads at the top, two at each side, and two at the bottom. Secure to the frame so that the specimen is in the exact center.

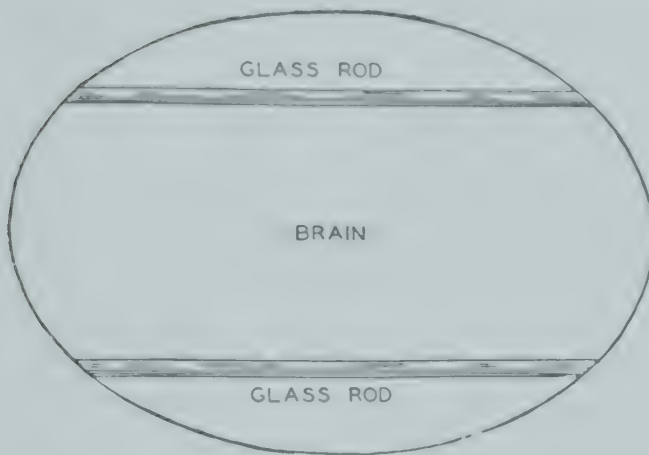


Fig. 128.—Diagram of brain braced with two concealed glass rods.

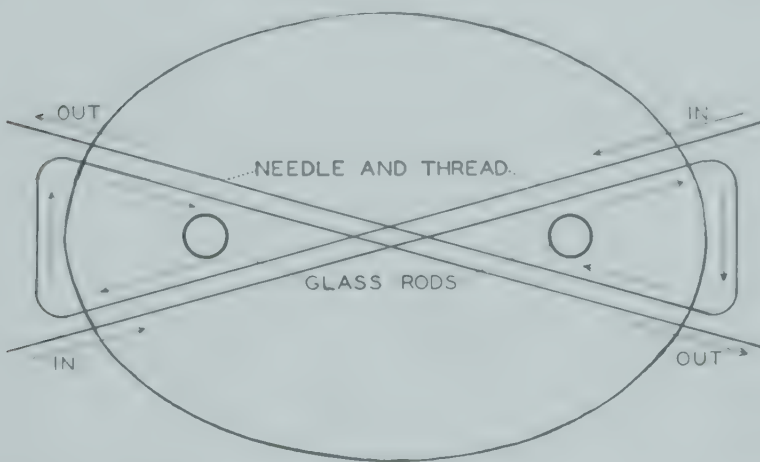


Fig. 129.—Diagram of cross-section of brain showing method of placing threads around the glass rods.

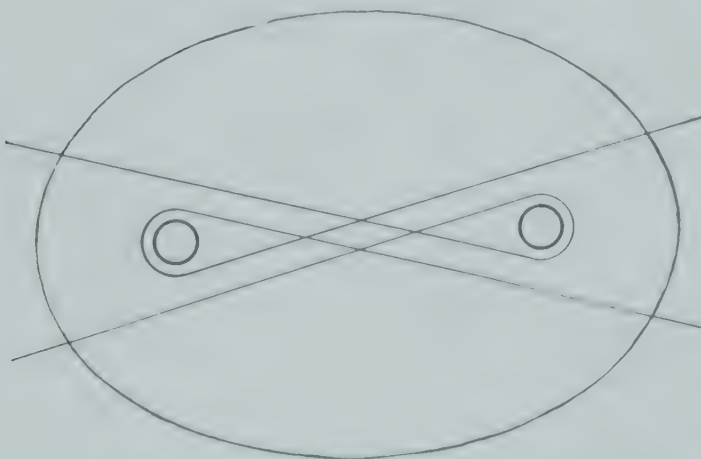


Fig. 130.—Diagram of cross-section of brain showing threads drawn up around the glass rods.

For a series, use the same technic, placing one specimen below the other in a tall jar, and tying off in the same fashion.

When mounting a kidney, bring all stitches to the blood vessels and anchor them, or they will tear out.

Center a specimen by placing it in the frame in such way that it is equidistant from the top and bottom, and also from the two sides. In the museum, do not have one specimen up at the top of the jar and the next at the bottom of the jar, but all in the centers of the jars. When centering, allow for the thickness of the jar, and also the thickness of the cement seal.

If you have to have a very fine thread with a lot of strain on it, use surgical silk instead of cotton thread. If this is too heavy, unroll the silk and use one strand. This is *very seldom* used.

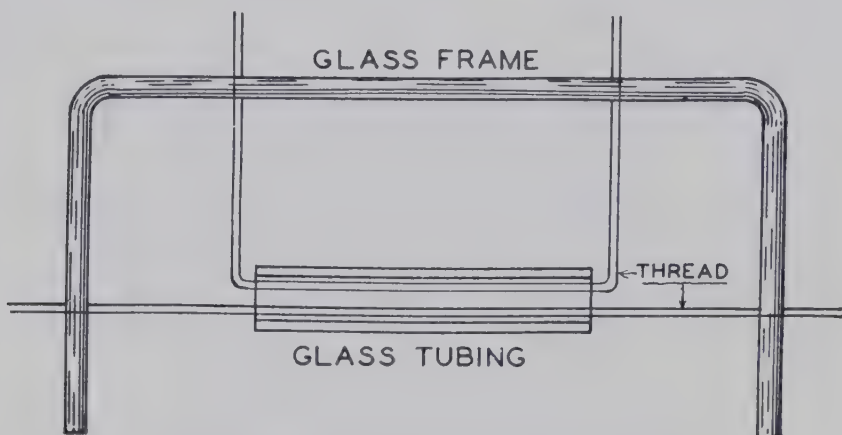


Fig. 131.—Method of mounting soft tissue.

The Sealing of Museum Jars*

In sealing museum jars, it is necessary to use a cement which will hermetically seal on the cover, and at the same time permit of its easy removal, but which will also undergo contacts with fluids without being dissolved. Muir and Judah have found the following formula to give best results. It will withstand such fluids as alcohol, formalin, and Kaiserling No. III, and will allow for necessary expansion and contraction.

Trinidad Lake asphalt	-----	1,200 Gm.
Boiled linseed oil	-----	170 c.c.
Crude oil of amber (not the refined)	-----	30 c.c.

*Condensed from Bulletin No. 5 of the International Association of Medical Museums. William Muir and Ernest Lionel Judah, Medical Museum, McGill University, Montreal.

Heat oils in quart enamel saucepan, slowly adding 400 Gm. of finely broken asphalt. Allow to boil half an hour. Cool. Add remainder of asphalt, 200 Gm., at a time, and boil for 30 minutes, cooling between each addition. Skim off surface debris. Boil mixture for 45 minutes until hard enough to use; that is, when only a slight impression is made on the surface, when cold, by a finger print. In very warm climates, it may be necessary to add 200 Gm. more of the asphalt, or to boil longer.

It is difficult, but possible, to boil down the mixture with only 600 Gm. of asphalt. This gives a more elastic, and therefore better cement, but often a skin forms, such as is found on partially dried linseed oil. This must be prevented.

Pour in small evaporating dishes enough cement to seal about twenty-five jars. This prevents overhardening.

If two wet surfaces are to be mounted together, as when mounting in fluid under Petri dishes, use a less firm cement, the consistency of which is judged by the ease of manipulating under water. Muir and Judah found the common Trinidad Lake asphalt better than the refined asphalt because of the higher degree of elasticity it possesses. Foreign matter usually precipitates out and is left in the pan.

Directions for Use

In the back left-hand corner of the cover, drill a small hole, just large enough to take a small cork. After filling the jar to within about an inch from the top, thoroughly clean both the top edge of the jar and the cover, to remove dirt or grease. Heat the cover on a thick asbestos plate, and keep it hot enough so that it can just be held in the hand. Move it around to heat evenly.

Heat the cement slowly in a sand bath until liquid, taking care not to allow it to smoke or burn. After heating the top edge of the jar with a Bunsen flame until it is just too hot for the hand, apply the cement with a knife, keeping the edges where the cement has not yet been applied hot with the Bunsen flame. Apply the cement in such a way that it will run down the outside, not the inside of the jar. After applying the cement to all four edges, reheat both the cement and the top of the jar, keeping the cement liquid, but do not burn it, and do not allow it to run down into the jar. Now put the hot cover onto the hot cement so that it is in proper position, and press down firmly so that all superfluous cement is forced to the outer edge.

While the cement is still hot, scrape off the surface until it is even with the jar. Allow to stand overnight, or until thoroughly cold. Clean with benzine. Apply a coat of varnish (Jap-a-lac) to the exposed surface. Do not apply any fluid to the jar until the cement has thoroughly set and is cold.

When the varnish is dry, fill the jar through the hole in the cover. The fluid should just reach the level of the cement but should not touch the cover. Seal the hole with a cork and a drop of hot cement.

Hand Specimens

Keep hand specimens in porcelain crocks with records as follows: (1) the name of the organ, (2) accession number, (3) hospital number, (4) surgical number, (5) short descriptive label. If this material is needed for demonstration purposes, remove it from the jar and send to the lecturer in a white pan. Label with ordinary gummed paper label, taking care that there is no grease under the label to form bubbles and subsequently cause the loss of the label. Brush heated hard paraffin across the label.

Museum Labels.—Cardboard labels may be used if desired, but since white cardboard turns yellow with age, tan cardboard is preferred. If color is desirable, make labels as follows: Place on the carbon side of red carbon paper a sheet of cellophane, then another red carbon paper, carbonated on both sides, then a sheet of onion-skin writing paper. Place this assembled material in the typewriter and write the label. Mount the colored cellophane between lantern slide glasses.

Inexpensive Watch Glass Museum Mountings*

(Modification of Klotz)

The method of mounting specimens for museum purposes should be of interest to many hospitals where the preservation of interesting pathological specimens is desired but where it is not possible on account of the prohibitive cost of some types of clock glass and museum jar mountings.

Description of the Method

A description of the preparation of a specimen from the time it is removed from the body until a finished mounted product is

*Larson, C. P., and Levin, E. J.: Museum Mountings at Minimum Cost, Arch. Path. 23: 536, 1937.

obtained is given. As a fixative use a modification of Klotz's No. 2 solution, because it preserves the natural colors better, with less bleaching, than any other fixative. An additional advantage is that the gross specimen is preserved in the same fluid in which it is finally mounted, thus obviating the necessity of time-consuming changes from one type of fluid to another. However, Kaiserling's or any of the routine fixatives may be used. The stock solution is prepared as follows:

Sodium chloride -----	1,000 Gm.
Sodium bicarbonate -----	1,800 Gm.
Sodium sulfate (crystals) -----	2,200 Gm.
Chloral hydrate -----	2,000 Gm.
Solution of formaldehyde, U.S.P. (40 per cent)	1,000 Gm.

Make up to 20,000 c.c. with water. To make routine fixative mix 2,000 c.c. of stock solution with 17,900 c.c. of water, and add 100 c.c. of acetone.

Directions: Make up solution of salts, formaldehyde, and water, and allow to stand. Crystalline sodium sulfate must be used, as the amorphous powder will not dissolve properly. Add the acetone when the batch is to be used.

Place the specimen in the fixing fluid, using a sufficient amount of the solution to equal ten times the bulk of the specimen. If the fluid becomes too deeply blood stained, change to fresh fluid. When the specimen is firm and the bleeding has ceased, it may be mounted. This usually requires from 10 to 30 days. Some types of gross material, such as thick slides of liver or spleen or entire organs, must be fixed in a more concentrated solution. Prepare this solution by using twice as much stock solution as was used in the routine fixative. It is wise to ascertain the desired shape of the tissue before fixing so that it may be placed into the fixing solution in such a way as to retain this form. For example, in the case of a strip of intestine, it may be laid out on a piece of white paper, to which it will adhere during the fixing and after which it will lay out flat with no support. Complete immersion of the gross material in the fixative is imperative to prevent any discoloration due to drying of the exposed surface. Hence, cover floating material with a moist towel and suspend heavy material by means of gauze.

A standard clock or watch glass of the desired size (a diameter of 8 inches, or 200 mm., will be suitable for many specimens) is then ground with emery powder so that the base is perfectly smooth and

holds a negative pressure when apposed to the glass base plate. Grinding may be done on a large sheet of plate glass, using first a coarse emery powder, such as grade 80, followed by a finer grade, such as emery flour or carborundum No. 400. Sprinkle the emery powder on the plate glass and wet with a little water. Grind by making sweeping circular motions of the clock glass on the grinding plate. About 5 minutes grinding with each grade of emery will suffice. When grating is no longer heard while polishing with the fine powder, a highly polished basal surface on the clock glass is obtained. This is the main essential as it is to be held to the base plate by negative pressure.

A suitable glass base plate is then selected, of a thickness depending on the diameter of the watch glass to be used. The reason for this is that the glass base plate assumes a concave shape after sealing, owing to the negative pressure formed by the contracting fluid. If the base plate is too thick, it will not be able to give under the strain of the contracted fluid. For the standard 5, 8, or 10 inch watch glasses (120, 125, 200, or 250 mm.) a base plate, with a thickness of $\frac{1}{8}$ inch (0.32 cm.), is best; ordinary double-strength or double-diamond window glass serves the purpose admirably. The glass base plate should not have any scratches on its surface and should be cut 2 inches (5 cm.) wider than the diameter of the watch glass.

The fluid used for mounting (solution described or other desired solution) should be brought to a boil in the same pan that is used for mounting in order to get as much air as possible out of the fluid. Allow the fluid to cool to approximately 100.4° F. (38° C.). The mounting pan should be porcelain so that there will be no chemical reaction between the solution and the metals of the pan. It should be sufficiently wide and deep to allow easy manipulation of the specimen, clock glass, and glass base plate. Place these articles into the fluid gently, without causing any air bubbles.

Put the specimen into the fluid and run the hands over it to free air bubbles which may be adhering to it. Slide the clock glass under the specimen, with the base upward, and orient the specimen so that it fits into the concavity of the watch glass in the desired position. Place one of the square edges of the base plate on the bottom of the pan against the edge of the clock glass, and gently lower the opposite edge of the base plate into the fluid so that any bubbles will rise up along the undersurface of this glass. Bring the

base plate into apposition with the whole undersurface of the clock glass, and, avoiding any undue pressure with the hands, lift the mount out of the fluid. Hold the mount up, shake it, and inspect for any air bubbles. If air bubbles still remain in the mount, immediately reimmerse it into the fluid with the clock glass down, and slide one edge of the base plate off the clock glass, allowing the air to escape. Then slide the base plate back into its proper position and remove the amount from the fluid.

The most rapid means of cooling the mount is to hold it under cold tap water; this seals the specimen almost instantly. Place the mount on a table and allow to dry for 2 or 3 days. If the base of the clock glass has been well ground, there will be no leaking of air into the mount. It is often necessary to clear the mount again because of the collection of air bubbles, which have probably been sucked out of the tissue itself. The clearing is best done after the mount has been allowed to stand for a few days, at which time most of the air will have left the tissue and collected in the form of a single bubble.

In order to clear this bubble it will be necessary to immerse the mount in a much warmer fluid than that of the original mounting temperature. The reason for this is that the slight evaporation which occurs during the drying process increases the negative pressure within the mount, making it very difficult to free the watch glass from the base plate. Hence, an effective means of separation is to allow hot tap water to run over the surface of the mount. Then plunge the mount into cold water; this will permit the sliding of the watch glass off the base plate. Clear the bubble and remount as previously described.

It is wise to prepare several gross tissues, watch glasses, and base plates before proceeding with the mounting. If this has been done previously, 20 specimens may easily be mounted in one afternoon.

The question arises as to how permanent and stable such a mount is under varying degrees of temperature. The clock glass will not drop off until the temperature in the surrounding air reaches a much higher level than the temperature of the fluid in which the original mounting was done. If the mounts have been allowed to dry for 3 days, they will stand a temperature of 145° F. (62.6° C.) before separation of the watch glass occurs. Some of these mounts have been tested in this manner by standing them upright in an incubator at this temperature for as long as 3 weeks at a time, with

no separation or sliding of the watch glass. The mount will also stand any temperature not lower than 40° F. (4.5° C.), but at a temperature lower than this there is danger that either the watch glass or the glass base plate will crack. The basic principle which makes this type of mount successful is that the glass base plate is thin enough to be able to accommodate the contraction of the fluid by buckling inward, forming a concavity on the exposed surface.

No additional sealing is necessary for temporary mounts, but if permanent preservation is desired, cement must be placed around the edge of the watch glass to prevent evaporation of the contained fluid. To accomplish this, apply any liquid cement with a needle and syringe around the edge of the watch glass. A very satisfactory and easy cement to apply is Paragon permanent adhesive. This cement is soluble in acetone, so it is wise to store the needle and syringe in this solvent.

To give the mount a finished appearance, bevel the edges of the base plate by polishing with emery before mounting the specimen.

Tagging Museum Specimens

It is vitally important to attach a reference number to the specimen, to give a clue to the clinical history and to the museum notes of the case. Tags must not rust or dissolve, nor must the identification of the specimen be lost. The method of W. G. MacCallum of Johns Hopkins Hospital, Baltimore, is excellent. Stamp consecutive numbers with a Bates automatic numbering machine on a length of white tape one inch wide, paraffin it, and roll on a stick. Cut off the number next in order, and attach to the specimen.

The following method is used at McGill University: Cut sheets of Bristol board 1 mm. thick into blocks 1½ cm. square, clip off the corners, writing the reference on the squares with waterproof ink. Punch a hole near the center, taking care not to interfere with the writing. Boil in hard paraffin half an hour. Remove with dissecting forceps, shake off superfluous paraffin, and drop in running water. Stitch loosely on the specimen so that the numbers are easily read from either side without danger of bending or breaking the block. Use as small a block as possible.

To identify the specimen, inscribe one side of the block with the entry number, putting the donor's name or hospital reference on the reverse side. Attach duplicate tags to every specimen. Store specimens until they are wanted in the museum.

Before mounting for the museum shelves, rewrite the tag with a small drawing pen, using as small and thin a block as possible, and boiling it in paraffin as above. Rub off superfluous paraffin with the thumb and forefinger, and attach to the back of the specimen in an inconspicuous place, using a very fine needle and thread. Tags should lie snug but not tight, and should be tied with a double surgical knot. The hole should be punched in the block before it is paraffined. Then remove the previously attached storage tags. The specimen is thus never without identification. With this method, the specimen is tagged with both the hospital and museum references. This is important for if fire occurs in the museum or if the jars are broken the hospital reference number will identify the specimen.

When paraffining gummed labels on museum jars, it is often difficult to make them adhere properly. Cleanse the jar thoroughly, and coat it with shellac. Apply label, allow to dry, then paint over with hot paraffin applied with a small flat paintbrush about one inch wide, taking care that the edges of the label are completely covered with paraffin. If the specimen is for the museum shelves, trim the label to within $\frac{1}{8}$ inch of its margin, and remove superfluous paraffin. Keep the labels clean with a cloth slightly moistened with benzine.

The Rusting of Metals

There is nothing that will prevent the rusting of metals. Remove any foreign body from the specimen and replace with glass. Break up an old bottle and make it into a foreign body and place in the specimen. Do not use metal or enamel dishes. Never use pins in a specimen unless that part of the specimen in which the pins were placed is later cut away. Preferably make glass pins or use tooth-picks or sew the specimen.

Laboratory Specimens

Alcohol, 50 to 60 per cent concentration, is a better preservative than formalin for specimens for microscopic work. They can still be stained after twenty years.

Proper Method of Taking Specimen for Microscopic Examination

1. Liver: Take a flat slab off the surface. (Same for any specimen already sliced.)

2. Intestine: Take out *lens*-shaped piece then close together. Cut straight through the specimen. Never take out a square piece.

3. Kidney, spleen, etc. Take out a "V"-shaped piece. It is never necessary to destroy a gross specimen by taking out a square piece.

Take specimen for microscopic examination *before specimen* has been placed in Kaiserling No. I.

Background for Specimens Mounted in Jars

Specimens mounted in museum jars are much improved in appearance when provided with a contrasting background. A convenient and permanent material for this purpose is Formica, similar to Bakelite, which may be obtained in various colors from the McClarin Taylor Company, Cincinnati, Ohio.

A sheet is sewed to fit, and placed inside the jar, back of the specimen. Black is usually most suitable. Orange may be used for dark specimens. Some specimens may be mounted by fastening them to Formica.

Staining the Fetal Skeleton

Fix entire fetus in 95 per cent alcohol for 4 days or longer. Then place in 1 to 3 per cent solution of potassium hydroxide (according to the size of specimen) until the bones are clearly visible. Should the specimen begin to fall apart, add a few drops of glycerin to the hydroxide solution. Siphon the potassium hydroxide off and cover the specimen with dilute solution of alizarin, 1 part in 10,000 of 1 per cent potassium hydroxide. Allow the stain to act until desired intensity is obtained. Then siphon the solution off, and add a solution of 79 parts of water, 20 parts of glycerin, and 1 part of potassium hydroxide.

Pass through increasing concentrations of glycerin, increasing 5 per cent each week, until in pure glycerin.

The success with this method depends on complete clearing before staining.

Method to Blacken Wooden Table Tops

Wooden table tops, white, or oak, are blackened permanently, using two solutions made up as follows:

Solution No. 1

Copper sulfate -----	125 Gm.
Potassium chlorate -----	125 Gm.
Water -----	1,000 c.c.

Boil until dissolved.

Solution No. 2

Aniline oil -----	100 c.c.
Hydrochloric acid, concentrated -----	93 c.c.
Water -----	1,000 c.c.

Apply one coat of No. 1 to the table tops with a brush, and apply a second as soon as the first is dry.

When the second coat is dry, apply in the same way two coats of No. 2 solution and let dry thoroughly.

Then apply a coat of raw linseed oil with a cloth and rub until well polished.

Repeat this process once a day for one week until the polishing rag no longer removes the blue green color of the copper sulfate.

To keep the table tops in good condition rub them with liquid petrolatum once a week.

The above process has been applied to stone (albarine) table tops with satisfactory results.

To protect the floor from blackening, spread papers under and around the tables.

Cleaning Slides and Cover Glasses

New slides and cover glasses are placed in 95 per cent alcohol for 15 minutes or longer. They are then removed, one at a time, and carefully dried with a soft lint-free towel or cloth. They are kept in tightly covered containers to protect them from dust and moisture.

Cleaning Mixture for Used Slides and Cover Glasses

Potassium dichromate -----	20 Gm.
Sulfuric acid, concentrated -----	75 c.c.
Water -----	100 c.c.

Dissolve the potassium dichromate in water by the aid of heat. Allow the solution to cool. Then very slowly pour in the acid, stirring the mixture constantly.

Used slides and cover glasses are soaked in xylol for a few days to remove the gum and balsam. The covers are then removed and placed in 95 per cent alcohol to remove the xylol. They are then washed in tap water and placed in the cleaning mixture for several days.

When thoroughly clean, they should be rinsed in several changes of water, allowed to stand in water weakly alkalized with ammonia, washed in running water for several hours, placed in 95 per cent alcohol, dried in usual manner, and stored in containers.

DILUTION OF 96 PER CENT ALCOHOL

GRADE REQUIRED	VOLUMES OF 96 PER CENT ALCOHOL	VOLUMES OF WATER
90 per cent	93.5	6.5
80 per cent	83.3	16.7
70 per cent	72.9	27.1
60 per cent	62.5	37.5
50 per cent	52.1	47.9
40 per cent	41.6	58.4
30 per cent	31.2	68.8

MANUFACTURERS AND DEALERS IN LABORATORY SUPPLIES

Manufacturers of Microscopes, Microtomes, and Accessories:

Bausch and Lomb Optical Company, Rochester, N. Y.

Ernest Leitz Optical Works, Wetzlar, Germany.

American representative: Ernest Leitz, Inc., 60 East 10th St., New York, N. Y.

Spencer Lens Company, Buffalo, N. Y.

Carl Zeiss Optical Company, Jena, Germany.

American representative: Carl Zeiss, Inc., 485 Fifth Ave., New York, N. Y.

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Merck and Company, Inc., New York, N. Y.

Biological Stains:

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Gradwohl Laboratories, 3514 Lucas Ave., St. Louis 3, Mo.

Hartman-Leddon Company, Inc., 5821 Market St., Philadelphia 39, Pa.

National Aniline and Chemical Company, Inc., New York, N. Y.

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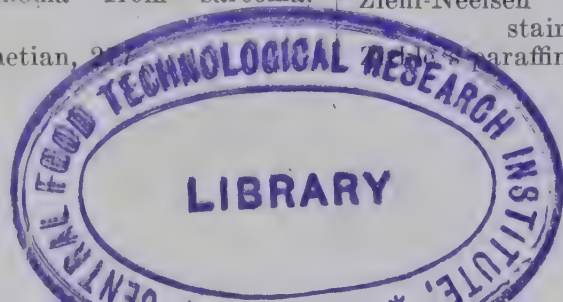
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